Enzymatic production and purification of prebiotic oligosaccharides by chromatography and membrane systems

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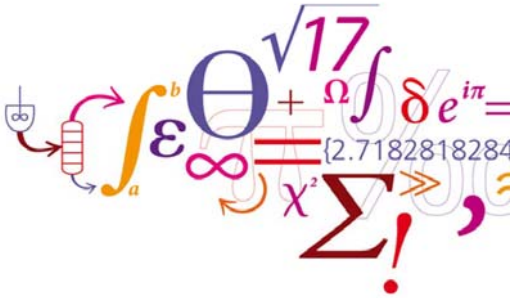

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Ph.D. Thesis

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PREFACE

This thesis comprises the research done during a PhD project carried out at the BioEng Center for Bioprocess Engineering, Department of Chemical and Biochemical Engineering, at Technical University of Denmark, from November 2008 to January 2014.

The work was accomplished under the supervision of professor Jørn D. Mikkelsen and cosupervision of associate professor Gunnar E. Jonsson and associate professor Manuel Pinelo.

The project was carried out within the Center for Biological Production of Dietary Fibers and Prebiotics at DTU ("Prebiotics Center") and as a part of "Enzymatic Production of Human Milk Oligosaccharides" project supported by The Strategic Research Council.

This thesis is submitted towards fulfilling the requirements for obtaining the degree of PhD at the Technical University of Denmark.

M. Michalak

Malwina Michalak

January 2014

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I would like to dedicate this work to my daughter Kika.

ABSTRACT

Enzymatic treatment of biomass is an environmentally friendly method to obtain a range of products, such as biofuels, animal feed or food ingredients. The objective of this PhD study was to produce functional food ingredients – oligosaccharides and polysaccharides by means of enzymatic catalysis from biomass: casein glycomacropeptide (cGMP) and potato pulp. These saccharides should possess prebiotic properties, i.e., they should be non-digestable, selectively fermented and allow specific changes, both in the composition and/or activity of the gastrointestinal microbiota that confers benefits upon host well-being. Therefore the obtained compounds were incubated with single bacterial cultures to examine their prebiotic potential.

Different types of oligosaccharides were produced in the present study. The first group of compounds was human milk oligosaccharides (HMOs) containing sialic acid in their structures. These were synthesized from cGMP (a donor of sialic acid) and appropriate glycan in trans-glycosylation reaction catalysed by mutant sialidase from *Trypanosama rangeli* expressed in *Pichia pastoris*, Tr6. Production of the model HMO, sialyllactose was enlarged to 5 L scale.

The second type of sialylated oligosaccharides was obtained with the same donor of sialic acid – cGMP and different glycans with a new *Trypanosoma rangeli* trans-sialidase, Tr13. Well-documented prebiotics galactooligosaccharides (GOS), iso-maltooligosaccharides (IMO) and lactulose, and three other compounds, i.e., melibiose, maltose, and fucose were sialylated with this enzyme resulting in creating novel human milk- like oligosaccharides. Both HMO and human milk- like oligosaccharides were purified by filtration and chromatography.

The last compounds produced during this study were GOS and some galactopolysaccharides. They were generated from isolated galactan and galactan contained in solubilised potato pulp polysaccharides (SPPP). An endo-1,4-β-galactanase from *Emericella nidulans* was produced in a recombinant *P. pastoris* strain to catalyse hydrolysis of galactan and SPPP. This enzyme was purified, characterized and its crystal structure was determined. The products of enzymatic hydrolysis were fractionated according to their molecular weight using membrane filtration.

The results of this work pave the way for development of new functional food ingredients from industrial side-streams and generate value-added products with valuable biological properties and great market potential.

DANSK SAMMENFATNING

Behandling af biomasse med enzymer er en bæredygtig grøn metode hvorved mange produkter, såsom biobrændstof, foder og fødevare ingredienser kan fremstilles. Målet med dette PhD studie var at producere funktionelle fødevare ingredienser – oligo-sakkarider og poly-sakkarider ved hjælp af enzymatisk katalyse af forskellige biomasser; casein glycomacropeptide (cGMP) og kartoffel pulp. Disse sakkarider har et prebiotisk potentiale, dvs. de er svære at nedbryde af mave/tarm floraen, de kan fremme eller hæmme mikroorganismerne og give en helbredsmæssig effekt. De forskellige produkter blev derfor testede i vækstforsøg med enkelt celle kulturer fra mave/tarm systemet.

I dette PhD studie blev der fremstillet en række af forskellige oligo-sakkarider. Den første gruppe var Humane Mælke Oligo-sakkarider (HMOs), der indeholder sialinsyre. Disse stoffer blev fremstillet ud fra cGMP (doner til sialinsyre) og forskellige acceptor molekyler (glycaner). Katalysen var Tr6, der er en muteret sialidase fra *Trypanosomas rangeli*, der har 6 mutanter og har en rimelig trans-sialidase aktivitet. Tr6 blev produceret i *Pichia pastoris*. Produktionen af sialyl-laktose blev udført i 5-L skala.

Den anden gruppe af sialylerede oligo-sakkarider blev fremstillet med den samme donor, cGMP, men med Tr13, et mutant enzym med 13 mutationer, og en meget høj trans-sialidase aktivitet. Ved rationel mutagenese fik vi vendt hydrolyse (sialidase) til en Transferase (trans-sialidase). Nogle af de godkendte prebiotiske oligo-sakkarider, Galacto-oligosakkarid (GOS), og isomalto-oligosakkarid (IMO), samt andre glycaner såsom melibiose, maltose og fucose blev sialyleret ved hjælp af dette Tr13 enzym. Alle produkterne blev oprenset vha. membran-filtrering og anion-kromatografi og deres struktur blev bestemt ved LC-MS og NMR. De nye sialylerede oligo-sakkarider blev navngivet som HMO-lignende molekyler.

Den tredje kategori af prebiotiske molekyler var galactooligo-sakkarider, der blev fremstillet ved enzymatisk katalyse fra kartoffel pulp galactaner også kaldet SPPP (”solubilized potato pulp polysaccharides”). Det anvendte enzym, en endo-1,4-β-galactanase fra *Emericella nidulans* blev produceret i en rekombinant *P. pastoris* stamme, og blev oprenset ved søjle kromatografi, karakteriseret, hvorefter 3-D strukturen blev bestemt.

Resultaterne in denne PhD Tese viser en ny vej til fremstilling af nye funktionelle fødevare ingredienser fra industrielle side-strømme og værdiskabende nye produkter med stor marked potentiale.

LIST OF PUBLICATIONS

The thesis is based on the work described in the following publications:

Michalak M., Larsen D.M., Jers C., Almeida J.R.M., Willer M., Li H., Kirpekar F., Kjærulff L., Gotfredsen C.H., Nordvang R.T., Meyer A.S., Mikkelsen J.D.; Biocatalytic production of 3'-sialyllactose by use of a modified sialidase with superior trans-sialidase activity.; Process Biochemistry, http://dx.doi.org/10.1016/j.procbio.2013.10.023.

Jers C., Michalak M., Larsen D.M., Kepp K.P., Li H., Guo Y., Kirpekar F., Meyer A.S., Mikkelsen J.D.; Rational design of a new *Trypanosoma rangeli* trans-sialidase for efficient sialylation of glycans.; PLoS ONE, e83902. doi:10.1371/journal.pone.0083902.

Michalak M., Thomassen L.V., Roytio H., Ouwehand A.C., Meyer A.S., Mikkelsen J.D.; Enzyme and Microbial Technology, 2012, 50: 121- 129; Expression and characterization of an endo-1,4-ß-galactanase from *Emericella nidulans* in *Pichia pastoris* for enzymatic design of potentially prebiotic oligosaccharides from potato galactans.

Otten H., Michalak M., Mikkelsen J.D., Larsen S.; Acta Cryst., 2013, 69: 850-854; The binding of zinc ions to *Emericella nidulans* endo-ß-1,4-galactanase is essential for crystal formation.

Publications not discussed in the thesis:

Gavlighi H., Michalak M., Meyer A.S., Mikkelsen J.D.; J. Agric. Food Chem., 2013, 61: 1272-1278; Enzymatic depolymerization of gum Tragacanth: Bifidogenic potential of low molecular weight oligosaccharides.

Holck J., Larsen D.M., Michalak M., Li H., Kjærulff L., Kirpekar F., Gotfredsen C.H., Forssten S., Ouwehand A.C., Mikkelsen J.D., Meyer A.S.; New Biotechnology, 2014, 31: 156-165; Enzyme catalysed production of sialylated human milk oligosaccharides and galactooligosaccharides by *Trypanosoma cruzi* trans-sialidase.

Mikkelsen J.D., Jers C., Michalak M., Kepp K.P., Larsen D.M.; A mutant sialidase having trans-sialidase activity for use in production of sialylated glycans; patent application no.: 13163551.8-1410

Meyer A.S., Gavlighi H.A., Mikkelsen J.D., Michalak M., Ale M.T.; Enzymatic production of polysaccharides from Gum Tragacanth; patent application no.: 13164552.5-1501

ABBREVIATIONS

cGMP	casein glycomacropeptide
DP	degree of polymerization
E/S	enzyme to substrate ratio
FOS	fructooligosaccharides
GOS	galactooligosaccharides
HMOs	human milk oligosaccharides
HPAEC	high-performance anion exchange chromatography
IMO	iso-maltooligosaccharides
LC/MS	liquid chromatography/ mass spectrometry
LNFP I	lacto-N-fucopentaose I
LNFP V	lacto-N-fucopentaose V
LNnT	lacto-N-neotetraose
LNT	lacto-N-tetraose
MU-Gal	4-methylumbelliferyl-β-D-galactopyranoside
MW	molecular weight
NMR	nuclear magnetic resonance
PG	potato galactan
pNP-NeuAc	*para*-nitrophenyl neuraminic acid
RGI	rhamnogalacturonan I
SCFA	short-chain fatty acids
SPPP	solubilsed potato pulp polysaccharides
TcTS	*Trypanosoma cruzi* trans-sialidase
Tr6	*Trypanosoma rangeli* mutant sialidase with 6 point mutations
Tr13	*Trypanosoma rangeli* mutant sialidase from Tr6 parent with 7 point mutations

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1 HYPOTHESES AND OBJECTIVES

This PhD study was focused on enzymatic synthesis of oligo- and polysaccharides from side-streams from the food industry. The produced compounds are potential functional food ingredients. They were fractionated or purified by membrane filtration and/or column chromatography. Their biological properties were examined by measuring growth stimulation of gut bacteria in prebiotic tests.

General hypotheses of this work:

- It is possible to use side-streams from dairy and agriculture to generate value-added products, i.e. food fibers, like oligosaccharides and polysaccharides.
- Saccharides of different structures may be purified by column chromatography or separated into different molecular weight fractions by membrane technology.
- Oligosaccharides produced from side-streams possess prebiotic properties.

Objectives of this work:

- To produce and characterize enzymes for optimal catalysis of oligo- and polysaccharides production.
- To produce value-added products from industrial side-streams.
- To fractionate or purify oligo- and polysaccharides.
- To examine prebiotic potential of obtained products.

2 THEORETICAL INTRODUCTION

2.1 FUNCTIONAL FOOD INGREDIENTS

The concept of functional foods was introduced in Japan in middle 1980s. Its objective was to improve the health of the population and thereby reduce the health-care expenses of the society. In 1991 this type of food was legislated in Japan as “foods for specified health use” (FOSHU). In the same year oligosaccharides as fructo-, galacto-, soybean and palatinose oligosaccharides were listed as FOSHU. Until 1996 FOSHU status embraced also lactulose, lactosucrose, xylo- and isomaltooligosaccharides. The term “functional foods” first appeared in 1993 in the “Nature” magazine under the heading “Japan explores the boundary between food and medicine” (Henry, 2010).

Functional food is generally not recognized by law, besides Japan. It is essentially a marketing term. The Food and Drug Administration (FDA) regulates claims that manufacturers make about foods' nutrient content and effects on disease, health or body function. The FDA regulates these types of foods according to whether a food is considered to be a conventional food, a food additive, a dietary supplement, a medical food or a food for special dietary use.

There are several definitions of functional food which are presented in Table 1. Functional food can be: (1) natural food, (2) a food to which a component is added, both by increasing concentration of a component naturally occurring and by adding a component that is not naturally present (e.g. fructooligosaccharides), (3) a food from which a component is removed (e.g. allergenic protein), (4) a food where one or more components are modified, (5) a food in which the bioavailability is modified and (6) any combinations of mentioned above (Henry, 2010).

The functional food defined by different institutions and Japanese food for specific health use define the same criteria that should be met: (1) this type of food should be a part of usual diet, (2) it should be in food form, (3) it should contain an ingredient which provides health benefit (Duncan et al., 2012; Farr, 1997). The Japanese Ministry of Health, Labour and Welfare mentions also more features of FOSHU: the health benefit of the food or relevant components should have a medical or/and nutritional basis, the appropriate level of consumption should be definable based on medical or nutritional knowledge, the food or relevant components should be safe and well defined in terms of physicochemical properties and methods of determination, it should not be the components exclusively used in medicaments, the composition of the product should not be notably defective in comparison with the composition of nutritive components that are normally contained in similar types of food (Farr, 1997).

organization	definition
Japanese Ministry of Health, Labour and Welfare - Foods for Specified Health Uses (FOSHU)	Foods for Specified Health Uses (FOSHU) refer to foods containing ingredient with functions for health and officially approved to claim its physiological effects on the human body. FOSHU is intended to be consumed for the maintenance or promotion of health or special health uses by people who wish to control health conditions.
Health Canada	A functional food is similar in appearance to, or may be a conventional food, consumed as part of a usual diet, and is demonstrated to have physiological benefits and/or reduce risk of chronic disease beyond basic nutritional functions.
Food and Agricultural Organization (FAO) of the United Nations	Functional foods should be a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of the usual diet which contains biologically active components with demonstrated physiological benefits and offers the potential of reducing the risk of chronic disease beyond basic nutritional functions.
Academy of Nutrition and Dietetics (formerly the American Dietetic Association)	All foods provide some level of physiological function, but the term functional food is defined as whole foods along with fortified, enriched, or enhanced foods that have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels.
Dieticians of Canada (DC)	Functional foods are foods that offer unique health benefits that go beyond simply meeting basic nutrient needs. Many also help to reduce chronic disease risk. Functional foods contain "bioactive compounds," or naturally occurring chemicals that act on our bodies. It is these bioactive compounds that offer the health and wellness benefits that have been linked to functional foods.
International Food Information Council (IFIC)	Functional foods are foods or dietary components that may provide a health benefit beyond basic nutrition.
International Life Sciences Institute of North America (ILSI)	Functional foods are foods that by virtue of physiologically active food components provide health benefits beyond basic nutrition.
Ministry of Agriculture, Fisheries and Food in United Kingdom	Functional food is a food that has a component incorporated into it to give a specific medical or physiological benefit, other than a purely nutritional effect.

Table 1. Functional food definitions, adapted from Duncan et al., 2012; Henry, 2010; Farr, 1997.

The assessment of safety of the potential functional food is proposed to be based, in some cases, on comparison with traditional acceptable reference food. When the functional food is substantially equivalent or similar to the known traditional food, the nutritional and toxicological studies may be reduced (Farr, 1997).

Among the functional food ingredients many different types of compounds are mentioned: phytosterols lowering the blood cholesterol level by preventing its absorption from the intestine, antioxidants playing fundamental role in prevention of cancer by neutralization of free radicals, polyunsaturated fatty acids showing an activity in prevention of cardiovascular diseases, as well as oligosaccharides, dietary fibers, probiotics, prebiotics and synbiotics.

2.2 OLIGOSACCHARIDES

Oligosaccharides are used in food for different purposes. They are often used as prebiotics. However, some oligosaccharides are susceptible to hydrolysis in small intestine and therefore they do not exhibit prebiotic properties. That does not exclude them from application in food. Maltooligosaccharides are, for example, susceptible to hydrolysis but they still demonstrate ability to reduce levels of harmful bacteria in the gut. Maltooligosaccharides are produced from starch by debranching with pullulanase and isoamylase and then by hydrolysis catalysed by α-amylase (Crittenden and Playne, 1996). Cyclodextrins are another group of oligosaccharides used as functional food but not exhibiting prebiotic activity. They are cyclic maltooligosaccharides consisting of 6-12 glucose monomers. They are obtained from starch in the reaction catalysed by cyclomaltodextrin glucanotransferase. They can form inclusion complexes with various compounds by incorporating them into their structure. Cyclodextrins may therefore be used in stabilization of compounds, especially volatile ones, in protection of unstable compounds and in masking the substances causing bitterness in foods and medicines (Crittenden and Playne, 1996). They are also applied in water treatment to encapsulate and adsorb contaminants and in cosmetics, like toothpaste, fabric softeners, tissues to control the release of fragrances (Mussatto and Mancilla, 2007).

Most of the food-grade oligosaccharides may be used as sweeteners, like for example, glucosyl sucrose produced from maltose and sucrose by glucanotransferase. It does not cause tooth decay and suppress crystal formation and browning reactions which make it a good replacement of sugar (Crittenden and Playne, 1996).

2.3 FIBERS

The following definition of dietary fiber was proposed by AACC Dietary Fiber Definition Committee in 2001: “Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine

with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects, including laxation, and/or blood glucose attenuation".

Some specific examples of dietary fibers are pectin and their derivatives, cellulose, β-glucans, hemicelluloses and resistant starch. Cellulose can be found in general in plants. Hemicellulose is obtained mainly from oat, rye, wheat or barley. Pectin is extracted from fruit (e.g. citrus fruit) or vegetables (e.g. sugar beet). Different dietary fibers may be classified according to solubility in water, viscosity in aqueous solutions, gel-forming capabilities, and fermentation rate by the gut microflora. For example, pectin is known for its gelling properties and is widely used in many foods to modify the texture of the products.

Dietary fibers are associated with improvements of bowel function, reduced risk of gastrointestinal disorders, as well as reduced risk of colon cancer. They also decrease risk of cardiovascular diseases and type 2 diabetes, improve glycemic control, and reduce inflammations. Moreover, dietary fibers are associated with lowering lipid levels and increase of satiety, and therefore are recommended to help with weight loss.

There is just one step from dietary fibers to prebiotics and that is a selective stimulation of the growth or activity of health-promoting bacteria. The fibers do not stimulate selectively prebiotic bacteria in the colon, whereas prebiotics do. The majority of dietary fibers is constituted by polysaccharides, whereas prebiotics are mainly represented by oligosaccharides.

2.4 PROBIOTICS

Probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit to the host". The microorganisms must meet safety, functional and technological criteria in order to be applied as probiotics. They must not be pathogenic, should not have any connection with diarrheagenic bacteria and no ability to transfer antibiotic resistance genes. They should also maintain genetic stability and should not produce any toxic compounds. Furthermore, the microorganisms must survive the acidity, the bile and the digestive enzymes. Another attribute they should posses is antagonistic activity towards pathogens. From the technological point of view probiotic microorganisms should survive such processes as freeze-drying, spray-drying, stirring which causes hydrostatic pressure and mechanical stress to the cells. Moreover, in a bioreactor, bacterial growth is connected with quick nutrient depletion and accumulation of harmful metabolites in the bacterial culture. This has to be overcome by the cells and the bacteria should keep their viability. The probiotics should keep their properties during long shelf-storage and have good sensory properties (Grajek et al., 2005).

The most common probiotics include strains of *Lactobacillus* and *Bifidobacterium*. They are mainly used to produce yoghurts and capsules containing dried probiotic cultures. Use of probiotcs is associated with stimulation of immune system, treatment of gastrointestinal disorders, like constipation, diarrhoea and inflammatory bowel disease. Furthermore, some investigations indicate that probiotics may have effect against infection with *Helicobacter pylori* and prevent allergies, reduce risk of colon cancer, lower serum cholesterol and levels of faecal enzymes, which are potential mutagens and may induce tumors (Ziemer and Gibson, 1998). An article by Ouwehand et al., 1999 adds to this long list of health benefits also improvement of bioavailability of food compounds and production of vitamins. In publication by Chauhan and Chorawala, 2012 probiotics are ascribed even more beneficial properties. These are: alleviation of lactose intolerance, treatment of food allergies and prevention of necrotizing enterocolitis, which is a relatively often disease in premature infants.

Prebiotics are compounds that are non-digestable by human and selectively stimulate the growth of health-beneficial bacteria in the gut (more detailed description of these ingredients will follow in the chapter 2.5). The action of pro- and prebiotics can be combined and the food additive containing these two ingredients is called synbiotic. Combining pro- and prebiotics should result in an improved survival and implantation of healthy microorganisms in the host. One of the most obvious choices to create a symbiotic is to combine fructo- or galactooligosaccharides with a strain of *Bifidobacterium*, which is able to grow well on these substrates. Obviously, application of synbiotics should show the same effects as particular pro- and prebiotics applied. It could be also expected that combining these two functional food ingredients could enhance the health-beneficial effect. The literature reports that synbiotics have anticancer activity (Gibson et al., 2004), anti-pathogenic activity (Sheela and Suganya, 2012) and many others characteristic for probiotics and prebiotics. An effect that is not mentioned so often in case of pro- and prebiotics used alone is prevention of infections in people who underwent surgeries (Chauhan and Chorawala, 2012).

2.5 PREBIOTICS

2.5.1 Definition and determination

The most recent definition of prebiotics describes them as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health”.

The International Scientific Association for Probiotics and Prebiotics (ISAPP) describes the requirements for prebiotic effect as follows:

1) resistance to gastric acid, hydrolysis by mammalian enzymes and gastrointestinal absorption

2) fermentation by intestinal microorganisms

3) selective stimulation of the growth and/or the activity of intestinal microbiota associated with health and well-being.

Besides these three official requirements to be fulfilled in order to call compounds prebiotics, ISAPP mentions two additional ones, i.e., testing in human trials and administering in sufficient amounts to confer a measurable benefit.

Prebiotics target the microbiota which is already present in the host's ecosystem. Whereas probiotics deliver microbial supplements, which positively influence the host health by improvement of the composition of the colonic microbiota. This effect may, however, not be a long-term, since the probiotic bacteria need to compete against established microbial community in the gut. On the other hand, if there is a deficiency of the health-promoting microbiota in the large intestine, which may be caused by the disease or antibiotic treatment, then there is no point in administering prebiotics, because they cannot work effectively in such an environment.

In the publication by Gibson et al., 2004 the methods of determination of prebiotic properties of compounds are summarized. Testing non-digestibility comprises of *in vitro* testing resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption. *In vivo* experiments are performed by measurement of the recovery in faeces of an oral dosage. These tests are done either in human or in germ-free rats.

Testing if a carbohydrate can be fermented by intestinal microflora is also performed by *in vitro* or *in vivo* methods. The *in vitro* methods are performed in batch or continuous culture fermentation systems. Batch systems are inoculated with pure culture or cultures or with faecal slurry. Continuous systems are multi-chamber and reproduce characteristics of different gastrointestinal fragments. *In vivo* methods can be studied in humans or animals by addition of investigated substances to food. Then the measurement of the recovery of carbohydrate is done in case of humans. The animals are killed and the content of the gastrointestinal fragments is analyzed. Human studies may also be performed by measurement of exhaled air and the concentration of gases, in particular H_2, after oral application of single dose of the carbohydrate. The H_2 is produced by the bacteria present in the human colon.

Selective stimulation of growth of bacteria in the colon is assessed, for example, by use of pure culture studies, fluorescence *in situ* hybridisation (FISH), and direct community analysis (Gibson et al., 2004). Pure culture studies are rapid and straightforward but do not include cross-feeding effect between different strains. FISH can be used for culturable and non-culturable bacteria. The direct community analysis by PCR enables investigation of diversity of the entire sample.

2.5.2 Structures and production methods

Most of food-grade oligosaccharides are manufactured using enzyme-catalysed processes. They are either produced in trans-glycosylation reactions, e.g. lactosucrose, or as a result of hydrolysis of polysaccharide, e.g. fructooligosaccharides. Only soybean oligosaccharides are produced by direct extraction from plant. Lactulose is produced chemically from lactose in an alkali isomerization process.

The most extensively tested and confirmed prebiotics are lactulose, inulin, FOS and GOS (also called trans-galactooligosaccharides: transGOS). According to publication by Gibson et al., 2004 only these compounds deserve prebiotic status. The other sources, e.g. Charalampopoulos and Rastall, 2009 mention also xylooligosaccharides, mannan-derived compounds, arabinogalactans, resistant starch, iso-maltooligosaccharides, soybean oligosaccharides, lactosucrose, pectin and pectin-derived products as prebiotics. There is an increasing list of potential prebiotics which have not been as extensively studied as GOS or inulin, such as glucooligosaccharides, gentiooligosaccharides, palatinose oligosaccharides, raffinose, and lactitol. The structures and production methods of some established and potential prebiotics will be described in this chapter.

Lactulose (ß-D-Gal-(1,4)-ß-D-Fru) is manufactured from lactose (ß-D-Gal-(1,4)-α-D-Glc) in the process of isomerization in alkali solution. The glucose moiety in lactose is converted to fructose (Figure 1). Another compound with potential prebiotic activity produced from lactose is lactosucrose (ß-D-Gal-(1,4)-α-D-Glc-(1,2)-ß-D-Fru). It is produced in an enzymatic process involving ß-fructofuranosidase catalysing trans-fructosylation of lactose at its glucose residue at position C^1, creating a non-reducing oligosaccharide (Figure1). The co-substrate in enzymatic synthesis of lactosucrose is sucrose (α-D-Glc-(1,2)-ß-D-Fru) (Crittenden and Playne, 1996). The third product generated from lactose by transglycosylation with ß-galactosidase is a mixture of galactooligosaccharides (Figure 1). The structures, detailed production methods and prebiotic properties of galactooligosaccharides will be described in chapter 2.6.

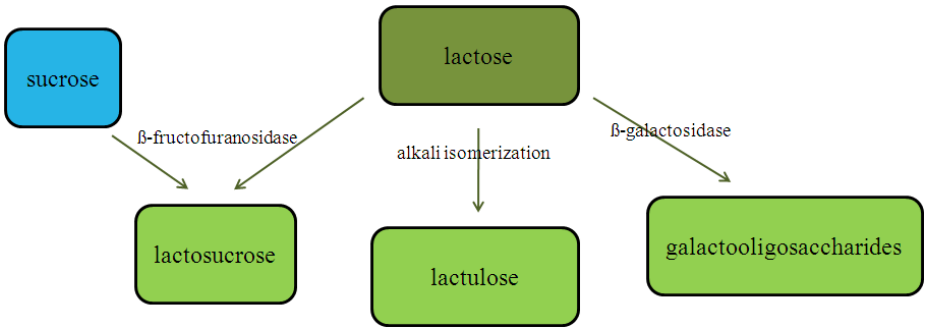


Figure 1. Oligosaccharides produced from lactose, adapted from Crittenden and Playne, 1996.

Sucrose, in similar manner to lactose, is a substrate for synthesis of prebiotic oligosaccharides. One of them was mentioned above: lactosucrose. Transfructosylation of sucrose with ß-fructofuranosidase (e.g. from *Aspergillus niger*) leads to creation of

mixture of fructooligosaccharides with DP from 2 to 4 (Figure 2). Glucose, fructose and un-reacted sucrose are removed from the product by chromatography. The action of dextran sucrase on sucrose and maltose results in synthesis of glucooligosaccharides (Gibson et al., 2004) (Figure 2).

Raffinose is a trisaccharide built of galactose, fructose and glucose (α-D-Gal-1,6-α-D-Glc-(1,2)-β-D-Fru). It can be found in beans, cabbage, brussels sprouts, broccoli, asparagus and some other vegetables. It can be synthesized from galactose and sucrose by means of α-galactosidase (Nakata et al., 2013) (Figure 2).

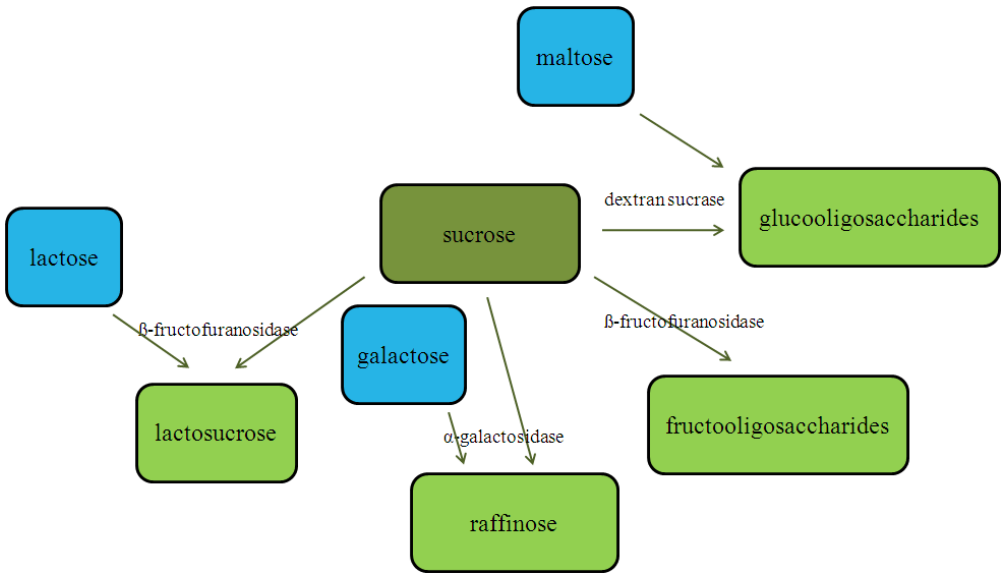


Figure 2. Prebiotic and potentially prebiotic oligosaccharides manufactured from sucrose.

Chicory inulin is composed of a mixture of oligo- and polysaccharides in which DP varies from 2 to 60 and the average DP is 12. By applying separation technologies a long-chain inulin may be produced with DP from 10 to 60 and average DP of 25. The partial hydrolysis of inulin by means of an endo-inulinase produces mixture of fructooligosaccharides in which DP varies from 2 to 7. Both inulin and fructooligosaccharides contain fragments consisting of α-D-glucopyranosyl-(β-D-fructofuranosyl)$_n$-β-D-fructofuranoside and β-D-fructopyranosyl-(β-D-fructofuranosyl)$_n$-β-D-fructofuranoside. Different industrial products vary in DP and properties (Gibson et al., 2004). As mentioned above fructooligosaccharides can also be generated from sucrose by action of β-fructosidase.

Gentiooligosaccharides ((ß-D-Glc-1,6)$_n$) are produced from glucose by enzymatic transglucosylation. Substrate for this reaction – glucose is obtained by enzymatic or acid hydrolysis of starch. Starch is also a substrate for generation of iso-maltooligosaccharides (Figure 3). Starch is liquefied by α-amylase and ß-amylase (both α- and ß-amylases act on α-1,4 bonds) to produce maltose. Maltose is subsequently used by α-glucosidase to produce iso-maltooligosaccharides by transglucosylation and converting α-1,4 bond to α-1,6 (Crittenden and Playne, 1996). The final product consists

of a mixture of oligosaccharides of DP from to 2 to 5, and different types of linkages, α-1,6 and α-1,4.

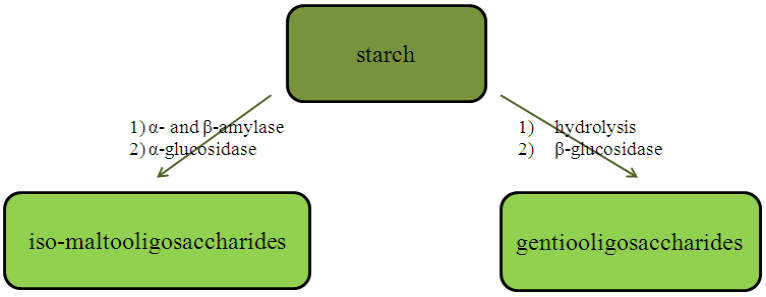


Figure 3. Oligosaccharides manufactured from starch, adapted from Crittenden and Playne, 1996.

Soybean oligosaccharides are directly extracted from the raw material, which is a soybean whey, a side-product from the production of soy protein. The saccharides extracted from this material comprise of raffinose (α-D-Gal-1,6-α-D-Glc-(1,2)-β-D-Fru), stachyose (α–D-Gal-1,6-α-D–Gal-1,6-α-D-Glc-1,2-β-D-Fru), sucrose, glucose and fructose. Raffinose and stachyose are not digestable and they can stimulate growth of bifidobacteria in the colon (Crittenden and Playne, 1996).

Pectins are complex heteropolysaccharides with gelling, thickening and emulsifying properties, which promote human health as dietary fibers (Gulfi et al., 2007). They are widely used in the food industry, in products such as jams, jellies, yoghurts and desserts. Almost all plants contain pectin in their cell walls. Especially citrus peels and sugar beet pulp are used as sources to extract pectin. The main component of pectin is homogalacturonan, which is built of α-1,4-linked galacturonic acid. The other component is xylogalacturonan, which is homogalacturonan substituted with single xylose residues at position C^3. Rhamnogalacturonan I (RGI) is the third pectic polysaccharide and its structure is very complex. The backbone of RGI is built of repeating disaccharide [→4)- α-GalA- (1,2)- α-Rha-(1→]. The rhamnose residues of RGI can be substituted at O^4 with neutral sugar side chains, composed mainly of galactosyl and/or arabinosyl moieties (galactan, arabinan, arabinogalactan). The side chains may vary from a single residue to more than 50. The last component of pectin is rhamnogalcturonan II, which has a backbone as homogalacturonan and complex side chains attached to the galacturonic acid residues. The side chains contain such rare moieties as, for example, apiose and aceric acid (Seveno et al., 2010). Mild acid hydrolysis of pectin leads to depolymerization of homogalacturonan. Alkaline conditions result in the removal of methyl and acetyl groups, as well as β-elimination reaction. Hydrothermal treatment leads to production of oligomers of galacturonic acid (Charalampopoulos and Rastall, 2009). There is a wide range of enzymes applied in modification of pectin. They are: pectin methyl esterase, pectin acetyl esterase, pectin lyase, pectate lyase, endo-polygalacturonase, exo-polygalacturonase, RGI lyase, RGI hydrolase, endo-xylogalacturonan hydrolase, RGI rhamnohydrolase, RGI galacturonohydrolase. An example of oligosaccharides generated from pectin are

homogalacturonides and rhamnogalacturonides, which were successfully produced by use of a sequence of monocomponent enzymes and separated by anion exchange chromatography, as described by Holck et al., 2011. There are many enzymes able to produce oligosaccharides from RGI side chains, e.g. endo-1,4-β-galactanase from *Emericella nidulans*, which action will be thoroughly described in chapters 6 and 7.

Xylooligosaccharides are produced by enzymatic hydrolysis or chemical treatment of xylan contained in different raw materials, like corn and sunflower hulls, olive pulp, straws, bagasses and bran. Xylan is a ubiquitous polysaccharide present in plant cell walls. Usually it is made up of a main backbone of β-1,4 xylose which is often substituted at positions C^2 and C^3 with arabinose, 4-O-methylglucuronic acid, acetyl or phenolic groups. The hydrothermal treatment of raw material results mainly in the products of DP between 9 and 25. Xylooligosaccharides of low DP can be obtained by application of endo-β-1,4-xylanases. In order to produce food-grade xylooligosaccharides, xylose and polysaccharides are removed by ultrafiltration, nanofiltration and precipitation with ethanol, acetone or 2-propanol (Charalampopoulos and Rastall, 2009).

2.5.3 Biological activities

The human gastrointestinal system consists of mouth, oral cavity, esophagus, stomach, small intestine and large intestine (colon). Biological functions of the large intestine are absorption, secretion of electrolytes and water, storage and excretion of waste materials. The functions that affect the health and well-being are connected, to some extent, to the gut microbiota (Gibson and Reberfroid, 1995), especially that large intestine is the most colonized part of digestive tract. To reach the large intestine compounds cannot be digested in any of the previous part of the digestive system. When the food ingredient reaches the colon it is fermented by the large intestinal microbiota.

Bacteria present in the colon are able to produce a wide range of compounds, which may have health-beneficial or negative effect on the host. The negative influence may be caused by, for example, production of cancerogenic compounds and toxins, such as ammonia, amines and phenols, which are products of peptides degradation (Brownawell et al., 2012). The compounds that may affect the host in a positive manner are, for example, short-chain fatty acids (SCFA).

Majority of human large intestinal microorganisms is strict anaerobes, and facultative anaerobes are many magnitudes lower. The dominant anaerobes are Bacteroides (Gram-negative rods) which may constitute up to 30% of the total faecal flora and have significant impact on processes in the colon. The other important groups are bifidobacteria, eubacteria, clostridia, and lactobacilli which are all Gram-positive rods. Bacteroides may exhibit health-promoting properties, such as stimulation of immune functions, promotion of minerals absorption, synthesis of vitamins, but also pathogenic effect, i.e. production of carcinogens. Eubacteria are associated with inhibition of pathogen growth. Bifidobacteria and lactobacilli are associated only with health-

beneficial effects, whereas clostridia are seen only as producing toxins pathogens (Gibson and Roberfroid, 1995).

Present prebiotics, fulfilling the requirement of selective stimulation of the growth of health-beneficial bacteria, are directed towards bifidobacteria and (to a lesser extend) lactobacilli. Lactobacilli may inhibit the growth of harmful bacteria, stimulate immune functions and enhance absorption of minerals (Gibson and Roberfroid, 1995). Bifidobacteria produce SCFA, acetate and lactate as metabolic end products. They lower the pH of the environment and thus exert an antibacterial activity towards pathogens (Kawase, 1982). Moreover, bifidobacteria contribute to lowering of blood cholesterol and ammonia levels and help to absorb minerals. They produce vitamins of group B and act as immunomodulators, e.g. by promoting attack against malignant cells (Gibson and Roberfroid, 1995).

The ability to stimulate production of short-chain fatty acids by prebiotics is evaluated very positively. Increased SCFA production is associated with improvement of immunity system and cancer prevention. For example, an intravenous administration of acetate increases peripheral blood antibody production and natural killer cells (a type of lymphocyte) activity in cancer patients compared to controls (Ishizaka et al., 1993). Butyrate stimulates apoptosis and may be a protective factor in carcinogenesis (Macfarlane et al., 2008). Almost all prebiotics or potential prebiotics were tested in relation to the immunological and anti-cancer effect (e.g. FOS, GOS, xylooligosaccharides, different pectin derived oligosaccharides) and all exhibited promising results. For example, propionate produced from GOS showed anti-inflammatory effect in respect to cancer cells (Nurmi et al., 2005). Furthermore, SCFA are an important source of energy for the host and they play a role in colonic epithelial cell transport processes, hepatic control of lipid and carbohydrate metabolism (Charalampopoulos and Rastall 2009). Modulation of immune system takes place, as stimulation of SCFA production, by stimulation of the growth of immunomodulatory bacteria (bifidobacteria and lactobacilli). In numerous investigations a mixture of GOS and FOS revealed the ability to stimulate the amount of faecal bifidobacteria and reduce the incidences of diseases in infants and children.

Experimental investigations in animals, and a few human studies, have shown interesting cholesterol and/or triglycerides lowering effects by applying prebiotics. Different mechanisms of influence on lipid metabolism are postulated. One involves lower capacity to synthesize lipoprotein in the liver, another decreasing fat digestibility. The ability to modulate lipid metabolism was mainly studied with FOS, but xylo- and manooligosaccharides demonstrate activity against triglycerides, phospholipids and cholesterol, too (Charalampopoulos and Rastall 2009). However, these effects may be also induced by selective growth of bacteria in the colon. Some studies report that relative proportions of Bacteroidetes to Firmicutes present in the large intestine are lower in obese than in lean people. Following the appropriate diet results in reduction in a group of butyrate-producing Firmicutes and increased relative abundance of Bacteroidetes (Brownawell et al., 2012; Duncan et al., 2012).

Another significant health effect of prebiotics on mammalian physiology is the ability to improve calcium, zinc, iron and magnesium absorption, which can be beneficial in preventing osteoporosis and avoiding diet-related anaemia (Charalampopoulos and Rastall 2009). Mineral absorption is connected to degradation of phytic acid, and for that prebiotics are responsible as well. Phytic acid molecule may posses up to six phosphate groups, which may bind mineral ions making them unavailable for absorption in the body. Therefore phytate is associated with reduced zinc and iron uptake. Studies in rat indicated that prebiotic consumption enhanced degradation of phytic acid, because SCFA produced in bacterial fermentation induce phytases. Moreover, SCFA form soluble ligands with cations, which prevent their binding to phytic acid (Charalampopoulos and Rastall, 2009). On the other hand, degradation of phytic acid may not be so beneficial because it possesses anti-carcinogenic activity (Shamsuddin, 2002).

Other often mentioned health benefits associated with prebiotics are: increase of numbers of epithelial cells and the intensity of their secretory functions (Charalampopoulos and Rastall, 2009), laxative effect (Macfarlane et al., 2007), reduction of rheumatoid arthritis by altering inflammatory joint symptoms (Charalampopoulos and Rastall, 2009), antihypertensive and lowering blood glucose effects (Chauhan and Chorawala, 2012). Prebiotics are also widely tested against inflammatory bowel disease (IBD), a multifactorial disorder associated with reduced levels of bifidobacteria and increased levels of putatively pathogenic microorganisms. Prebiotic action in IBD includes the bifidogenic effect and immunomodulation (Charalampopoulos and Rastall, 2009).

Properties of prebiotics were extensively tested in the context of infants health. Studies in allergic infants show reduction in the incidence of atopic dermatitis, which is associated with increased numbers of bifidobacteria (Moro et al., 2006). Furthermore, bifidobacterial colonization stimulated by prebiotics reduces a risk of necrotizing enterocolitis which is one of the most common causes of morbidity in premature infants (Charalampopoulos and Rastall, 2009). The bifidobacteria and SCFA patterns in infants fed with formula containing prebiotics are similar to these that are breast-fed. The prebiotic-containing formulas have also favourable effect on stool consistency. However, there are still questions to be answered, e.g. prebiotics may reduce the risk of disease but should they be used in case of illness? Moreover, the optimal composition of prebiotics and their dosage should still be studied further, and more studies *in vivo* should be done (Veerneman-Wauters, 2005). More information is needed concerning the growth, body composition, nutrient availability and water balance in infants fed with prebiotics (Macfarlane et al., 2007).

The consumption of prebiotics has a variety of metabolic consequences. Many of physiological traits attributed to prebiotics are related to the effects of gut microbiota and its biochemical activities. The majority of investigations looked at only a few bacterial groups. The growth effects seen in these studies may not be replicated in the gut, where many different carbon sources are available for the bacteria. In summary: in

order to confirm prebiotic effects, well conducted human trials are required. That is the most reliable way to properly evaluate the properties of these ingredients, since only the human body offers the very complex *in vivo* conditions which enable occurrence of numerous processes, like bacterial cross-feeding effect, SCFA release and absorption at the same time, decrease in toxins release, expression of different enzymes, minerals absorption, changes in mucosal structures, altered lipid metabolism, rise in bifidobacteria or lactobacilli amount, decrease of pathogens growth and stimulation of the immunity.

2.5.4 Applications and market

Some prebiotics occur naturally in foods, for example, in artichoke, leek, chicory, asparagus, garlic, onion, oat, tomatoes and soybean. Unfortunately their content is low and it would require large amount of these vegetables to evoke a prebiotic effect. More practical method of observing prebiotics effects is to enrich food products with defined amount of prebiotics. Currently prebiotic compounds are added to products such as cereals, yoghurts, breads, biscuits, jellies, ice-creams, spreads (such as jams and marmalades), drinks and infant formulas. They can also be applied in non-food products e.g. as drug delivery basis. Moreover, there are advantages provided to product formulas by the physicochemical properties of prebiotic oligosaccharides, such as increased viscosity or protection from dryness. For example, xylooligosaccharides are applied in cosmetics as moisturizing agents (Charalampopoulos and Rastall 2009). They may also be applied as bulking agents because of their reduced sweetness (usually 0.3-0.6 times as sweet as sucrose) or in low-caloric food because of their indigestibility and high viscosity in the solutions. Additionally, prebiotic oligosaccharides can be used to control the amount of browning due to the Maillard reactions in heat-processed foods. Furthermore, future prebiotics may promote growth of other than *Bifidobacterium* and *Lactobacillus* beneficial gut bacteria, for example, *Eubacterium*, *Propionibacterium*, *and Roseburia*. All these features ensure that production of prebiotics will continue to expand in the future.

Prebiotics are added to infant formulas in an attempt to conserve or restore high bifidobacteria content in the microflora, since high bifidobacteria content prevents from infections and allergies. The prebiotics used in infant formulas are usually GOS and inulin (sometimes FOS). They are applied at concentrations up to 0.8 g/dl, since the Scientific Committee on Food of the European Commission stated that addition of the prebiotic mixture at a concentration of 0.8 g/dl to infant formula is considered safe (Veerneman-Wauters, 2005).

The regulatory status for carbohydrate prebiotics on the market today is Generally Recognized As Safe (GRAS). The European Union regulates prebiotics as Novel Foods. Prebiotics, including inulin and FOS, were submitted for approval of a health claim but were rejected. Therefore, it is unlikely that the European Food Safety Authority (EFSA) will permit any health claim for prebiotics in the near future, just as it has disallowed any health claims for probiotics (Sloan and Hutt, 2013).

Nutrition Business Journal estimated the gastrointestinal supplement market at $1.5 billion in 2012, +13% vs. 2011. After cholesterol lowering effect, digestive health was the second most purchased condition-specific food/beverage category in 2011 (Sloan and Hutt, 2013). Therefore, new and novel prebiotics will likely be the future drivers of the market. Especially that new food and beverages with prebiotics are growing quickly in the global market (291 in 2012 vs. 195 in 2011).

In general, food-grade prebiotic oligosaccharides are mixtures of oligosaccharides of different degree of polymerization. Often the substrate, i.e. polysaccharide or monosaccharide is present as well. Most manufacturers produce different classes of products with different purity level. The production of food-grade oligosaccharides except lactulose was 35000 t in 1991 and increased to 65000 t in 1995. Lactulose production in 1995 was estimated to be about 20000 t, whereas GOS production was 15000 t and FOS 12000 t (Crittenden and Playne, 1996).

The manufacturers of animal feed and pet food started to show interest in prebiotics as well. The main aim of application of prebiotics in these products is the reduction of infections and decrease of faecal putrefactive compounds. In case of animal feed the intension to introduce prebiotics, concerns outbreak of food-borne diseases, which has mainly been caused by prohibition of antibiotics as growth promoters (Gaggia et al., 2010). Including prebiotics in the chickens and cattle diet does not have a strong health-improving effect. Application of synbiotics is more beneficial for both groups of animals. It improves gut health and control pathogen release in the environment, decreasing the risk of food-borne infections in humans. (Gaggia et al., 2010). Incorporating fructooligosaccharides into dogs' diet resulted in the increase of number of bifidobacteria and increase in concentration of short chain fatty acids. The other outcome of supplementing dogs with FOS was decrease in faecal concentration of ammonia and phenols which, among other compounds, are responsible for the malodour of dog faeces (Hussein et al., 1999). Introducing FOS to canine diet induced only to a low extent increase in bifidobacteria, but glucose-based oligosaccharides caused a significant increase (Gibson et al., 2004). As can be seen, the nutrition research for companion animals generates broad perspectives for prebiotics use.

It can be concluded that the prebiotics production will continuously increase and more applications and forms for these food ingredients will appear in the future. The scientific investigation towards new prebiotic compounds, as well as better understanding of their mode of action will definitely accompany the trends in the industry and the market.

2.6 GALACTOOLIGOSACCHARIDES

2.6.1 Methods of production

Galactooligosaccharides (GOS) are prebiotic food ingredients. The commercial method of production of galactooligosaccharides involves lactose as substrate, as both, donor

and acceptor in the reaction. Lactose is relatively cheap and moreover it may be a side-product from production of, e.g. lactose-free milk. The conversion of lactose to GOS is performed in enzyme-catalysed reaction. The enzymes employed for this purpose are bacterial and fungal galactosidases – glycoside hydrolases, which act as glycoside transferases, e.g. β-galactosidase from *Bacillus circulans* used in production of GOS by Friesland Campina or *Aspergillus oryzae* and *Streptococcus thermophillus* β-galactosidases used by Yakult Honsha. The literature reports broad range of enzymes producing galactooligosaccharides from lactose, e.g. α-galactosidase from *Aspergillus nidulans* (*Emericella nidulans*) (Nakai et al., 2010), β-galactosidases from different strains of *Bifidobacterium*: *B. angulatum*, *B. bifidum*, *B. adolescentis*, *B. infantis* and *B. pseudolongum* (Rabiu et al., 2001), β-galactosidase from *Lactobaciulls reuteri* (Maischberger et al., 2007), β-galactosidase from *Kluveromyces lactis* (Chockchaisawasdee et al., 2005) and many others.

GOS are produced as mixtures of oligosaccharides of different DP, usually from 1 to 9, since the mixture contains also un-reacted lactose and free glucose and galactose. This happens because GOS are simultaneously synthesized and degraded by β-galactosidase. The degree of polymerization depends on microbial origin of enzyme, substrate concentration and reaction conditions. *A. oryzae* β-galactosidase shows optimal product formation at pH 3.5, whereas yeast and bacterial enzymes have pH optima around 6-7.5 (Charalampopoulos and Rastall, 2009). Furthermore, by pH manipulation, the trans-galactosylation can be promoted and hydrolysis can be kept low. Higher temperature speeds up the reaction. Moreover, high temperature is preferred because of better lactose solubility and prevention of its crystallization, as well as reduction of reaction mixture viscosity when high substrate concentrations are used. High substrate concentration is favoured because it results in decrease of water activity and thus in decreased hydrolysis and production of GOS of higher DP. Therefore the thermostable β-galactosidases are getting more attention (Onishi and Tanaka, 1995; Park et al., 2008; Nakao et al, 1994; Ji et al., 2005; Petzelbauer et al., 2000). Another factor influencing the DP of galactooligosaccharides is the reaction time. It often happens that prolonged incubation with the enzyme leads to hydrolysis of the products and therefore to decrease in the average DP. On the other hand, short reaction time results in higher DP, but also in lower conversion of the substrate.

Depending on the enzymes used in the production process there appear different glycosidic linkages in the structures of galactooligosaccharides, which influences, of course, the prebiotic and physicochemical properties of the product. For example β-galactosidases from *B. circulans* and *Cryptococcus laurentii* form mainly 4'-galactosyl lactose, whereas enzyme from *S. thermophilus* and *B. bifidum* 3'-galactosyl lactose. Fungal β-galactosidase from *A. oryzae* generates 3'- and 6'-galactosyl lactose and yeast enzyme from *K. lactis* 6'-galactosyl lactose (Charalampopoulos and Rastall, 2009). Moreover, depending on the enzyme applied in the process of GOS production, the monosaccharide composition of the products may vary. Usually GOS have a structure

of Gal_n-Glc, but there can be also structures Gal_n-Gal (where n means the degree of polymerization) or even branched structures (Torres et al., 2010).

The yields of GOS production are usually lower than 50%. In Table 2 the maximum GOS concentrations produced with different enzymes and different reaction conditions are presented. The yields of GOS have been found to be higher when using organic solvents which lower the water activity. The toxicity of organic solvents limits however their use in food production (Gosling et al., 2010).

Enzyme source	Reaction conditions			Lactose conversion (%)	Maximum GOS yield (% w/w)
	Initial lactose concentration [g/L]	T (°C)	pH		
Sulfolobus solfataricus	600	80	6.0	70	52.5
Sulfolobus solfataricus	270	70	5.5	72	26
Aspergillus oryzae	500	40	4.5	56	52
Bullera singularis	180	50	6.0	72	50
Saccharopolyspora rectivirgula	600	70	7.0	75	41
Sterigmatomyces elviae	200	60	5.0	63	39
Lactobacillus reuteri	205	37	6.5	80	38
Pyrococcus furiosus	270	70	5.5	80	33
Bifidobacterium longum	400	45	6.8	60	32.5
Kluveromyces lactis	400	40	7	92	24.8
Thermotoga maritima	500	80	6	50	19

Table 2. The maximum GOS concentrations; adapted from Gosling et al., 2010.

There are two types of reactors used for production of GOS. These are batch and continuous stirred reactors. In the continuous reactor, filtration happens at the same time as the reaction. In the batch process the enzyme applied in the reaction is usually lost after the termination of the reaction, whereas in the continuous process it may be reused and therefore decrease the product cost. Furthermore, the mode of operation influences the composition of produced GOS. In the batch system, the composition and concentration of galactosyl acceptors are changing constantly, while in the steady state

of a continuous system the concentration of acceptors stay constant (Charalampopoulos and Rastall, 2009). This leads to more defined GOS mixtures in case of continuous reactor (Splechtna et al., 2007), since it provides continuous removal of products and prevent their hydrolysis. Furthermore, the continuous process provides removal of glucose and galactose, which are known inhibitors of β-galactosidases (Gosling et al., 2010). The removal of monosaccharides increases therefore the yield of GOS production.

Another way of producing GOS involves whole cells. In that case there is no need to isolate the enzymatic catalyst. GOS yields up to 43% (w/w) have been achieved using this method and that makes it comparable with production using isolated enzymes (Gosling et al., 2010). Using whole cells have an advantage of removal of monosacchrides from the product, since growing microorganisms consume them in the process of growth. An example of employing the whole cells for production of galactooligosaccharides is use of mixture of *B. bifidum* and *Saccharomyces cerevisiae*. *S.cerevisiae* consumed 92% of glucose present in the reaction mixture and therefore purified the product (Gosling et al., 2010). The disadvantage of application of the whole cells is the presence of the metabolic end products, like ethanol or lactic acid.

When the reaction is conducted with isolated enzyme, the purification of GOS from substrate – lactose and monomers generated during the progress of the reaction is not an easy step. None of the methods used for that purpose provides complete separation of the desired GOS from side-products and lactose. It is however important for some applications of GOS to produce them in high purity. At first because many people are nowadays lactose intolerant and secondly because presence of monosaccharides limits applications in diabetic food. Removal of mono- and disaccharides produced from lactose has been attempted by different techniques. Comparison of them is described by Hernandez et al., 2009. Diafiltration with cellulose acetate membranes of cut off 500 and 1000 Da did not show any selectivity between mono-, di- and oligosaccharides. Activated charcoal treatment showed different selectivity in GOS recovery depending on the ethanol concentration in the solution in which Vivinal GOS were dissolved. The best results were obtained with 8 and 10% of ethanol. Applying the first concentration resulted in 90% recovery of GOS, but 20% of disaccharides were also recovered from active charcoal. 10% ethanol led to almost complete removal of disaccharides, but loss of about 47% of GOS. Another fractionation technique employed yeast. The commercial mixture of GOS was incubated with *Saccharomyces cerevisiae*, which removed monosaccharides selectively and converted them to ethanol and CO_2. The yeast did not, however, degrade the disaccharides. Another disadvantage of this method could be presence of the yeast's metabolites in the GOS solution. The most effective method of GOS fractionation was size exclusion chromatography. It separated almost completely mono-, di-, tri-, tetra- and pentasaccharides, and hexa-, hepta- and octasaccharides were obtained in high purity. Furthermore, the GOS recovery was high, i.e. between 81 and 92%.

Besides trans-galactosylation of lactose GOS may also be obtained by enzymatic hydrolysis of galactan (polymeric side chain of pectin rhamnogalacturonan I). Since galactan is built mainly of galactose monomers connected with 1,4-β, 1,6-β and 1,3-β linkages, the enzymes employed in its hydrolysis are endo-β-galactanases. Commercially available galactan contains besides galactose arabinose, galacturonic acid and rhamnose. Moreover, galactan is sometimes called arabinogalactan, usually in cases when the arabinose content is relatively high. The method of production of GOS (usually 1,4-β-GOS) from galactan is not applied in the industry. Until now it concerns mainly production of oligosaccharides in laboratory scale. However, due to the abundance of plant material, which is so far considered a waste stream from agriculture, this method has a potential for application in the big-scale production. The enzymes available for this purpose are getting more attention. There are fungal galactanases, e.g. from *Aspergillus aculeatus* (Luonteri et al., 2003) and *Aspergillus niger* (Yamaguchi et al., 1995; de Vries et al., 2002; Luonteri et al., 2003), as well as bacterial ones, e.g. from *Bifidobacterium longum* (Hinz et al., 2005), *Streptomyces avermitilis* (Ichinose et al., 2008) or *Thermotoga maritima* (Yang et al., 2006).

The endo-galactanases have different specificities and preferences towards different substrates. The endogalactanase from *B. longum* degrades 1,4-β and 1,3-β bonds between galactose units and it is hindered by the presence of arabinose in the substrate (Hinz et al., 2005). The β-1,4-endogalactanase from *A. niger* is able to produce galactooligosaccharides containing arabinose from soy arabinogalactan. The endo-β-1,6-galactanase from *A. aculeatus* was able to produce galactose and 1,6-β-galactobiose from 1,3/6-arabinogalactan, and moreover exhibited promiscuitism by liberating arabinose from arabinan (Luonteri et al., 2003). The endo-β-1,6-galactanase from *S. avermitilis* catalysed hydrolysis of β-1,6-linked galactosyl linkages of oligosaccharides and polysaccharides, as well as degradation of tomato and radish arabinogalactan protein (Ichinose et al., 2008).

The enzymatic hydrolysis of galactan, as transglycosylation of lactose, is not devoid of drawbacks. The main one is the presence of monomer in the product. Galactobiose is an important, if not the most abundant one, component of the products mixture too (Yamaguchi et al., 1995; Ichinose et al., 2008; Luonteri et al., 2003; de Vries et al., 2002). The content of galactose and galactobiose usually increases with the incubation time. For example, the endogalactanase from *B. longum* produces mainly galactotriose at the beginning of incubation with potato arabinogalactan and then degrades this trisaccharide to dimer and monomer (Hinz et al., 2005). Thus the composition of the GOS mixture may be influenced by choosing an appropriate reaction time. Application of another endo-1,4,-β-galactanase - from *Emericella nidulans* to obtain galactooligosaccharides and influence of time on product composition is described in the publication attached in chapter 9.3.

There is also a group of glycosyltransferases which can synthesize GOS. Although they are highly efficient, regio- and streoselective they are not used in industry due to their

poor availability and very high prices, as well as need of specific sugar nucleotides as substrates (Gosling et al., 2010).

Besides enzymatic reactions, GOS may be obtained by chemical methods, like acid hydrolysis of polysaccharides. An example of such a treatment is preparation of galactooligosaccharides exhibiting anti-angiogenesis activity from polysaccharides of *Nerium indicum* (Hu et al., 2009). Also sulfated galactooligosaccharides are often obtained by acid hydrolysis (Käsbaueret al., 2001), since it is troublesome to find enzymes active on sulfated polysaccharides.

2.6.2 Biological properties

The biological properties of GOS are the same as associated with prebiotics in general. Especially that the most investigated prebiotics are FOS and GOS. The most obvious biological activity of galactooligosaccharides as prebiotics is stimulation of probiotic bacteria. The probiotic strains which growth is usually tested are bifidobacteria and lactobacilli. GOS produced by enzymes of different microbial origin stimulate these bacterial strains to different extent. The best stimulation of bifidobacteria could be expected in case of GOS produced by bifidobacteria itself. In publication by Rabiu et al., 2001 β-galactosidases from different species of *Bifidobacterium* were used to produce GOS, which were subsequently tested on the same strains, as well as on *Lactobacillus acidophilus*. The highest single culture growth was achieved when the strains utilized GOS produced by their β-galactosidases. However, the mixed culture test revealed that the growth of probiotics was stimulated in some cases to the same extent by the commercially available product obtained by means of galactosidase from *A. oryzae*. On the other hand, it was demonstrated in human trials that GOS produced by β-galactosidase from *B. bifidum* had higher prebiotic potential than commercial GOS produced with β-galactosidase from *A. oryzae* (Gosling et al., 2010).

With stimulation of bifidobacteria are associated beneficial effects on human health, such as reduced amount of toxic metabolites, prevention of diarrhoea and growth of pathogens – mainly by induction of production of short chain fatty acids, and stimulation of the immune system (de Sousa et al., 2011). Moreover, many mineralization studies have been done with GOS. The galactooligosaccharides have been reported to stimulate the absorption of calcium and magnesium (Macfarlane et al., 2008; Weaver et al., 2011). Other positive influence of galactooligosaccharides on human body is demonstrated by ability to reduce incidence of allergies or alleviate joint inflammation (Macfarlane et al., 2008), as well as by the potential to protect from development of colorectal cancer (Charalampopoulos and Rastall, 2009).

GOS are nowadays often used in infant formulas in Europe. Their task in these products is to stimulate the growth of probiotic microorganisms, especially bifidobacteria and thus provide microflora similar to that possessed by breast-fed infants. The other effects that are supposed to be delivered by GOS are prevention of diseases and atopy, and stimulation of immune response.

2.6.3 Applications and market

Galactooligosaccharides have been manufactured and commercialized since 1980s. In 2007 the global market size was estimated to be about 20000 tones (Torres et al., 2010). The mixtures of GOS are produced, among others, by Yakult Honsha, Nissin Sugar Manufacturing Co. and Friesland Foods Domo. The commercial names of GOS mixtures produced by different companies and enzymes used for this purpose are presented in Table 3. The composition of GOS in different products is presented in Table 4.

company	product name	product form	enzyme source
Nissin Sugar Manufacturing Co.	Cup-Oligo	syrup or powder	*Cryptococcus laurentii*
Yakult Honsha	Oligomate 55	syrup or powder	*Aspergillus oryzae, Streptococcus thermophilus*
Friesland Foods Domo	Vivinal GOS	syrup or powder	*Bacillus circulans*
Clasado Ltd.	Bimuno	syrup or powder	*Bifidobacterium bifidum*
Corn Products Intl.	Purimune	powder	*Bacillus circulans*

Table 3. Commercial GOS available in the market; adapted from Torres et al., 2010.

As can be seen from Table 4 there are differences in the purity amongst the commercially offered GOS. The differences appear also in the linkages between monosaccharides and they are caused by application of different enzymes in the production process. The Oligomate GOS mixture contains mainly β1→6 linkages, Bimuno mainly β1→3 linkages, whereas Cup-Oligo, Vivinal GOS, and Purimune contain mainly β1→4 linkages (Torres et al, 2010).

The combination of GOS and inulin in a ratio of 9:1 is used in infant formulas. The concentrations up to 0.8 g/dL of these prebiotics may be used (Veereman-Wauters, 2005). Products such as Allomin by Semper, Nan by Nestle or Baby & Me Organic by Arla Foods contain GOS. Some of the infant formulas contain also probiotics, e.g. Nan by Nestle.

Besides infant milk formulas, GOS may be incorporated in other food products, such as beverages (e.g. fruit juices), flavoured milks, ice-creams, jams, confectionery products, and baked products. The physicochemical properties of GOS, like heat stability or moisture-binding capacity, make them desired food ingredients. For example, because of the physicochemical properties, bread is a suitable food for galactooligosaccharides inclusion. During the yeast fermentation and baking of bread, GOS are not broken and render bread excellent in taste and texture (Mussatto and Mancilla, 2007). Another

property of galactooligosaccharides is reduced sweetness comparing to sucrose. It is typically between 0.3 and 0.6 times less sweet than sucrose. They can be then added in relatively high amounts to food, which is required to exhibit prebiotic properties, and at the same time they may be applied in food for people with diabetes or overweight.

product	glucose	galactose	lactose	total GOS	DP of GOS
Cup-Oligo	25-30			70	-
Oligomate 55	18-39		10-22	50-60	DP2: 15-17; DP3: 18-24; DP4:10-16; DP>4: 205.4
Vivinal GOS	19-22	0.8-1.3	10-23	57-59	DP2: 19-27; DP3: 22-23; DP4: 11; DP>4: 6-7.6
Bimuno	18	12	22	48-55	DP2:25-29; DP3: 12-14; DP4: 6.7-7.7; DP>4: 3.8-4.4
Purimune	0-1	0-0.5	7-10	90-92	DP2: 16-21; DP3: 38-51; DP≥4: 25-29

Table 4. Composition of GOS products; adapted from Torres et al., 2010. Composition of GOS is given in % of w/w of dry matter.

Another interesting application of GOS is in cosmetics. As in food, their prebiotic properties may be used to stimulate growth of beneficial bacteria – this time on human skin (Torres et al., 2010). Moreover, GOS can be used in livestock feed, for example for the poultry, pigs or fish, as well as in the pet food (Torres et al., 2010).

2.7 HUMAN MILK OLIGOSACCHARIDES

2.7.1 Structures and methods of synthesis

Oligosaccharides are the third largest fraction of human milk, after lactose and lipids. The oligosaccharide content of human milk varies with the duration of lactation, diurnally and with the genetic makeup of the mother (Miller and McVeagh, 1999). The content of oligosaccharides in the milk is approximately 20 g/L in colostrum and 5 g/L in mature milk (Donovan, 2009). About 200 structures of human milk oligosaccharides (HMOs) have been identified (Ninonuevo et al., 2007). All HMOs have lactose core in their structures. Lactose is placed at the reducing end and it is elongated with N-acetyl-glucosamine units by different types of glycosidic linkages. Fucose and sialic acid are placed at the non-reducing end of HMOs molecules. The HMOs vary in size (from three to thirty two) and may be linear (designated as *para*-HMO) or branched (designated as *iso*-HMO). The majority of the HMOs is neutral and contains fucose (Macrobal et al., 2010). Some selected HMO structures are presented in Figure 4.

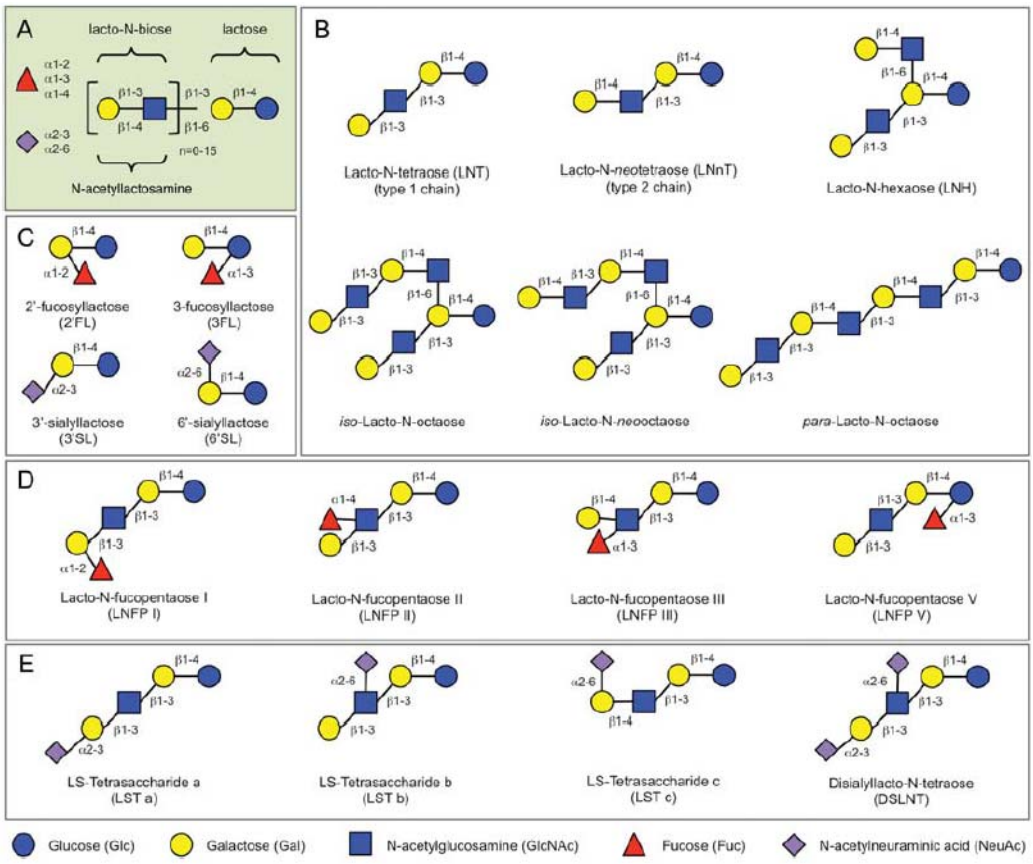


Figure 4. Selected HMO structures; adapted from Bode, 2012. A. HMOs blueprint; B. Lactose can be elongated by addition of either lacto-*N*-biose (type 1 chains) or *N*-acetyllactosamine (type 2 chains); C. Lactose can be fucosylated or sialylated; D. Elongated type 1 and 2 chains can be fucosylated; E. Elongated type 1 and 2 chains can be sialylated; monosaccharide key is shown at the bottom of the figure.

The HMO composition mirrors blood group characteristics, which depends on the expression of certain glycosyltransferases (Bode, 2012). Four milk groups can be determined by the activity of two gene loci encoding for the α-1,2-fucosyltransferase FUT2 (encoded by *Se* gene) and the α-1,3/4-fucosyltransferase FUT3 (encoded by the *Le* gene). Individuals with an active *Se* locus are classified as Secretors. Their milk is abundant in 2′-fucosyllactose, LNFP I and otherα-1,2-fucosylated HMOs. The non-Secretor lack a functional FUT2 enzyme and their milk does not contain α-1,2-fucosylated HMO. Individuals with an active *Le* locus are classified as *Le* positive. They express FUT3, which transfers fucose in the α-1,4 linkages to subterminal GlcNAc on type 1 chains. In contrast, the milk of *Le* negative women lacks these specific α-1,4-fucosylated HMOs (Bode, 2012).

According to publication by Bode, 2012 it is likely that HMOs biosynthesis is an extension of lactose synthesis, which occurs in the Golgi and starts with glucose. Moreover, there is a hypothesis that lactose core is extended by actions of *N*-acetylglucosaminyl-transferases and galactosyltransferases (Bode, 2012). Fucosylation

of HMOs occurs because enzyme FUT2 adds fucose in an α-1,2-linkage to terminal galactose, and FUT3 adds fucose in an α-1,4-linkage to internal *N*-acetylglucosamine. An additional unknown FUT enzyme adds fucose in an α-1,3-linkage to the reducing end glucose or internal *N*-acetylglucosamine (Bode, 2012). Thanks to that, even the milk of *Le* negative non-Secretor contains fucosylated HMOs. The biosynthesis of sialylated HMOs remains poorly understood (Bode, 2012).

The bovine milk which is a base for infant formulas contains very low amounts of the oligosaccharides present in human milk, less than 1 g/L (Jeong et al., 2012). The only known milk which is quite abundant in oligosaccharides of the same structures as human milk oligosaccharides, is milk of elephant. It is speculated that this occurs due to the fact that both man and elephant show similar patterns of postnatal ontogeny, i.e. they grow slowly, have highly developed nervous systems and show high degree of intelligence and learnt behaviour, as well as they are fed with the mother's milk for relatively long period (Osthoff et al., 2008).

The literature about synthesis of fucosylated oligosaccharides is quite limited. It reports that the synthesis of fucooligosaccharides can be catalysed by enzymes. The enzymes are often fucohydrolases which are genetically modified to exhibit trans-fucosidase activity (Osanjo et al., 2007; Wang, 2009). There are some microorganisms possessing α-fucosidases able to catalyse transfer of fucosyl moiety, for example *Alcaligenes* sp. (Zeng et al., 2003), *Corynebacterium* sp and *Aspergillus niger* (Ajisaka and Shirakabe, 1992). There are fucosidases of non-microorganism origin too, for example from canine and mollusc (Berteau et al., 2004). However, the enzymatic methods of producing fucosylated oligosaccharides are applied to obtain a very limited numbers and quantities of fucooligosaccharides. The enzymes are also often applied to catalyse reaction with a synthetic donor or/and acceptor of fucose, like *para*-nitrophenyl glycosides (Osanjo et al., 2007, Zeng et al., 2003). There are also general chemical methods of synthesis of different oligosaccharides. They require many protection and deprotection steps and may result in the presence of some harmful chemicals in the product. Therefore none of the mentioned methods may not be used in a large scale production of HMOs enabling incorporating these valuable compounds into infant formulas.

Sialylated oligosaccharides may be synthesized in vitro by chemical methods and enzymatic catalysis using sialyltransferases, trans-sialidases or sialidases. Chemical sialylation is usually complicated and time-consuming, since it requires multiple protection and de-protection steps (Yamamoto, 2010). Sialyltransferases transfer sialic acid (*N*-acetylneuraminic acid) from cytidine monophospho-sialic acid (CMP- sialic acid) to an acceptor. In this way they can synthesize sialyloligosaccharides, sialyl-glycoproteins and sialyl-glycolipids (Yamamoto, 2010). The disadvantage of sialyltransferases is the high price of the substrate. Sialidases hydrolyse sialylated glycans to sialic acid and glycan moiety. Their modification by mutagenesis may decrease the hydrolytic activity and rise their trans-sialidase activity. The transferase activity may be enhanced by application of proper reaction conditions, like in case of many lipases. Trans-sialidases catalyse the transfer of sialic acid from a donor to an

acceptor. Usually the donors of sialic acid are sialoglycoconjugates and the sialic acid is transferred to β-galactosyl groups in the acceptor molecules (Amaya et al., 2004). One of the widely-investigated trans-sialidases is *Trypanosoma cruzi* trans-sialidase (E.C.3.2.1.18). It is an enzyme naturally produced by the parasite responsible for Chagas disease (Augusti et al., 2007). It transfers α-2,3-bound sialic acid from the donor to terminal β-galactosyl moiety of an acceptor (Lee et al., 2002). The *T. cruzi* trans-sialidase expressed in *E. coli* is able to catalyse the transfer of sialic acid from fetuin, a glycoprotein from fetal calf serum, to lactose resulting in sialyllactose (Lee et al., 2002). Singh et al., 2000 reported that this enzyme also catalyses synthesis of sialylated oligosaccharides, including di-sialylated compounds. Moreover, Pelletier et al., 2001 employed *T. cruzi* trans-sialidase in production of sialyllactose from lactose and isolated κ-casein, as well as from lactose and 2,3-sialosides (such as κ-casein and gangliosides) contained in skim milk, mozzarella and Swiss cheese whey.

As mentioned above, sialidases can catalyse trans-sialidation. A patent by McJarrow et al., 2003 reported synthesis of sialylated saccharide from glycomacropeptide (GMP) and lactose by sialidases from *Arthrobacter ureafaciens* and *Bifidobacterium infantis*. Sialidases from *Vibrio cholera*, *Clostridium perfringens* and *Salmonella typhimurium* were applied to synthesize a series of sialyloligosaccharides using pNP-NeuAc as a donor, as reported by Schmidt et al., 2000. The examples of mutated sialidases exhibiting trans-sialidase activity are mutated *Trypanosoma rangeli* sialidases which contain five or six mutations (Paris et al., 2005). The *T. rangeli* sialidase is in 70% identical to *T. cruzi* trans-sialidase at the amino acid level. *T. rangeli* is not a pathogenic microorganism and therefore, from the commercial point of view, application of *T. rangeli* enzyme in potential production of sialylated compounds is more appealing than use of *T. cruzi* trans-sialidase. The applications of mutated *T. rangeli* sialidases in production of sialylated glycans are described in chapters 4 and 5, and in publications attached in chapters 10.1 and 10.2.

2.7.2 Biological properties

Infants digest a minor portion of HMOs present in the breast milk (Macrobal et al., 2010), because the glycosidic bounds between monosaccharides constituting HMOs cannot be hydrolyzed by lactase and other enzymes produced in human gastrointestinal tract. Lactase is not able to hydrolyze the lactose moiety of trisaccharides or longer oligosaccharides (Miller and McVeagh, 1999). Thus, most of the HMOs are delivered to the colon in an intact state and are treated as a carbon source by the colon bacteria. The HMOs are substrates for the probiotic bacteria, especially bifidobacteria. The presence of N-acetylglucosamine in the HMOs structures is necessary for the growth of *Bifidobacterium bifidum* in the large intestine (Miller and McVeagh, 1999). Two strains of *B. bifidum* (JCM1254 and JCM7004) are reported to possess exo-α-sialidase activity (EC 3.2.1.18) (Kiyohara et al., 2011). By the end of the first week of life bifidobacteria constitute 95% of the bacteria population in the faeces of breast-fed infants, whereas in the formula-fed infants they represent less than 70% (Miller and McVeagh, 1999). *Bifidobacterium longum* subsp. *infanti*s (ATCC15697) is considered an “archetypical

HMO-utilizing bacterium" (Sela et al., 2008). Macrobal et al., 2010 reports that *B. longum* subsp. *infantis* metabolizes HMOs with high efficiency and have specific preferences for fucosylated oligosaccharides.

The bifidobacteria produce short chain fatty acids which decrease pH in the colon and thus prevent growth of pathogens. Through this mechanism the HMOs decrease the incidence of diseases in newborns. For example, three fucosylated oligosaccharides representing the structures of HMOs: 2'-fucosyllactose, 3-fucosyllactose, and lactodifucotetraose stimulated the growth of two *B. longum* strains in mixed infant faecal bacterial communities. At the same time these bacteria produced lactate and short-chain fatty acids, which decreased the pH. *Escherichia coli* and *Clostridium perfringens* did not utilize the fucosylated oligosaccharides and the SCFA inhibited their growth (Yu et al., 2013). Moreover, the SCFA produced by the bifidobacteria are absorbed in the large bowel and therefore provide nutrition for the body. Therefore, most of the energy contained in the human milk oligosaccharides is finally available for the body of the infant (Miller and McVeagh, 1999). The stimulation of the growth of bifidobacteria by HMOs is beneficial also for another reason than production of SCFA. That is the production of vitamins, mainly of B group, by bifidobacteria (Gibson and Roberfroid, 1995). An interesting aspect of the HMOs is that their positive effect may also occur in the lactating mothers, since there was observed a "leakage" of the HMOs into their circulation (Bode, 2012).

Besides exhibiting the prebiotic properties, HMOs can stimulate bacterial growth in other manners according to Hunt et al., 2012. HMOs support the growth of *Staphylococcus aureus* and *Staphylococcus epidermis* without being consumed by these bacteria. The presence of HMOs stimulates bacterial growth and utilization of amino acids present in the medium. The HMOs may therefore promote the growth of *Staphylococcus* species in the lactating mammary gland and interact with the bacterial communities of lactating mother, and therefore extend their influence to the health of the lactating mother. On the other hand, *Staphylococcus* can also cause the breast inflammation (Bode, 2012). Since the *Staphylococcus* is present in mammary glands, it is also present in the human milk. Therefore, the bacteria in the breast milk can be regarded as natural probiotics, as well as commensals and pathogens.

HMOs modulate intestinal cell proliferation and maturation, which means that the mucosal barrier of the intestine can be affected by these compounds (Macrobal et al., 2010). HMOs may change the glycome of intestinal epithelial cells. 3'-sialyllactose reduces the expression of various glycosyltransferases, which diminishes the content of cell surface sialic acid, fucose and galactose (Bode, 2006). These modifications influence the biding ability of range of pathogens.

HMOs are perceived as elements playing a crucial role in the pathogen decoy mechanism. The mucosal surface in the human body contains receptors recognized by the pathogens. Since the HMOs structures are similar to the receptors in the epithelial cells, the bacteria may attach to HMOs instead. This prevents the infection, because the

bacteria are then "flushed out" from their potential host. The HMOs can by such a matter of action prevent diseases caused by dangerous pathogens like *Campylobacter jejuni* or *E. coli*, as well as viruses such as HIV and hepatitis C (Bode, 2006; Morrow et al., 2005). Sialyllactose, being one of the simplest structures of HMOs, inhibits binding of cholera toxin in vitro (Idota et al., 1995). Its exposure to Caco-2 cells reduces the adhesion of enteropathogenic *E. coli* by 50%, which is correlated with reduced cell surface sialic acid and lactosamine, which are key glycans for adhesion of enteropathogenic *E. coli* (Bode, 2006). Moreover, a disaccharide moiety of lactose or lactosamine most likely represents the core HMO recognition element for exotoxins expressed by *Clostridium difficile* (El-Hawiet et al., 2011). The similarities between oligosaccharidic ligands naturally occurring in human milk and the receptors for pathogens are listed in Table 5.

receptors	**microorganisms**
mannose-containing glycoproteins	*Escherichia coli* (type 1 fimbriae)
fucosylated oligosaccharides	*E. coli* (heat-stable enterotoxin)
fucosylated tetra- and pentasaccharides	*E. coli*
3'-sialyllactose, glycoproteins and sialyl-α-2,3-galactosides in mucins	*E. coli* (S-fimbriae)
neutral oligosaccharides (LNT, LNnT)	*Streptococcus pneumoniae*
gal-β-1,4-GlcNAc or Gal-β-1,3-GlcNAc	*Pseudomonas aeruginosa*
fuc-α-1,2-Gal epitopes	*Candida albicans*
sialyllactose	*Helicobacter pylori, Streptococcus sanguis*
sialyllactose and sialylated glycoproteins	*H. pylori*
sialylated glycoproteins (α-2,3-linked)	*Mycoplasma pneumonia*
sialylated poly-*N*-acetyllactosamine	*M. pneumonia*
sialylated α-2,3-poly-*N*-acetyllactosaminoglycans	*Streptococcus suis*
6'-sialyllactose	Influenza virus A
3'-sialyllactose	Influenza virus B
9-O-Ac of NeuAc-α-2,3-R	Influenza virus C

Table 5. HMOs as receptors for pathogenic microorganisms; adapted from Kunz et al., 2000.

The presence of sialic acid in the structures of many HMOs received also wide attention. The sialic acid-containing HMOs constitute about 20% of total HMOs (Wu et al., 2011). The sialic acid is a component of brain gangliosides. It is thought to play an essential role in nerve cell transmission, cell-to-cell communication and memory

formation (Miller and McVeagh, 1999). Thus, its availability in the infant food is an important factor in the postnatal brain development. The sialic acid is present not only in the brain but can also be detected in the saliva. The saliva was compared between breast-fed and formula-fed infants. This comparison resulted in detection of about 50% more sialic acid in the saliva of breast-fed neonates (Miller and McVeagh, 1999) indicating that the breast-fed neonates have higher content of sialic acid in their bodies. Sialic acid is also supposed to play a role in regulating immune response by serving as ligand for lectins (carbohydrate-binding proteins) binding (Wu et al., 2011). Furthermore, the sialylated oligosaccharides decrease leukocyte adhesion. The question arises: do HMOs compromise the infant's immune defence and harm the breast-fed infant or do HMOs keep the immune system in check and protect the breast-fed infant against overshooting immune response such as necrotizing enterocolitis (NEC) (Bode, 2006). Since the incidence of NEC is 85% lower in breast-fed infants than in formula-fed ones, it seems that the infant's own leukocytes and their excessive production of reactive oxygen species propagate NEC. Therefore, because the HMOs inhibit leukocyte adhesion at sites of inflammation, they may contribute to the protection against NEC (Bode, 2006).

The brain development requires, besides sialic acid as a component of gangliosides, also galactose which is a main saccharide in glycolipids in myelin. The liver may not be capable of providing all the galactose needed by the newborn mammals for the brain development and myelination. Therefore, a possible role of milk oligosaccharides in which galactose is a main component, is ensuring that galactose level in the infant do not become limiting during the brain development period (Kunz et al, 2000). A prerequisite for that is absorbtion of HMOs in the digestive tract without being digested. This phenomenon was confirmed in the study by Kunz et al., 2000. In that study ^{13}C-labelled galactose was administered orally to lactating mothers. That led to labelling of milk lactose and oligosaccharides in mammary gland, which enabled to follow metabolic pathways of labelled HMOs in the infants. The ^{13}C enrichment of milk was analysed by isotope ratio mass spectrometry (IR-MS). The highest content of ^{13}C was found in lactose, followed by neutral and, to a lesser extent, acidic oligosaccharides. Then the urine samples were collected from the infants fed with labelled milk. The urine contained ^{13}C and its highest content was found for lactose, followed by fucosyllactose, LNT, Fuc-LNT and Fuc_2-LNT. The presence of the HMOs in the urine indirectly proves their presence in the systemic circulation. The fact that some HMOs are absorbed without being digested in the gastrointestinal tract means that a variety of physiological effects of HMOs may be possible not only locally, but also after being absorbed.

2.7.3 Potential commercial applications

The potential commercial application of human milk oligosaccharides is obviously the infant formula. The fact that the compounds in the formula would be identical to the natural ones would make it an unbeatable product. Nowadays, the infant formulas contain galacto- and fructooligosaccharides, which do not naturally occur in human milk. The fructose monomer does not occur in human milk at all. GOS and FOS are not

sialylated or fucosylated, and presence of fucose and sialic acid is an important feature of HMOs. Addition of GOS and FOS to infant formulas stimulates the growth of bifidobacteria and provides some other benefits, but the long-term health benefits and risks of providing infants with significant amounts of these non-human milk glycans still need to be further investigated (Bode, 2012).

There could be more than one product created from HMOs. Depending on the dosage, for example, the product could be a food ingredient with disease preventive properties or a therapeutic medicine. Moreover, if the production of different types of HMOs would not be limited, the composition of the products containing HMOs could be differentiated. For example, the milk desired for newborns whose mothers are *Le* positive would contain oligosaccharides with fucose attached by α-1,4 linkage, whereas milk for newborns whose mothers are *Le* negative individuals would not contain such oligosaccharides. In such a manner the milk formula would be as similar as possible to the natural ones and "personalized". This last property could make it even more desired product for potential customers. Moreover, the HMOs could be used in combination with probiotics. The possibility to apply them in functional food for adults could be considered as well.

Until now, however, the possibilities of producing HMOs are quite limited. The milk of farm animals is not as complex as human milk. Therefore there are not any natural resources that could be used to obtain HMOs in the large amounts. To perform synthesis of different HMOs, there are several enzymes needed to catalyse this process, such as trans-sialidases and trans-fucosidases. The enzymes capable of attaching *N*-acetylglucosamine to oligosaccharide core would be useful as well. The β-galactosidases could also be employed in creation of HMOs. These enzymes are relatively well characterized, especially for the purpose of production of galactooligosaccharides used in infant formulas as HMOs substitutes. All the enzymes used for HMOs production should have food grade status which means that they should not have a pathogenic origin. The advantage of enzyme catalysis is that it is an environmentally friendly method, on the contrary to chemical methods. The chemical methods are, moreover, time-consuming and require multistep reactions. Furthermore, the application of enzymes enables utilization of side-products from food industry and that would provide double benefit by generating a product and, at the same time, avoiding the disposal of biomass. The use of the enzymes in the production process would ensure that no harsh chemicals are engaged and make the products safer for infants. Finally, the application of HMOs as the infant formulas ingredients requires, regardless of used production method, exhaustive clinical trials.

3 METHODOLOGY

This chapter describes experimental methods used in this PhD project.

PRODUCTION OF ENZYMES IN *P. PASTORIS* AND THEIR PURIFICATION

A 5 L Sartorius Biostat Aplus fermentor was inoculated with *P. pastoris* cells grown for 20 hours at 30°C, at 150 rpm in shaking flasks with minimal glycerol medium as detailed by Stratton et al., 1999. The 5 L scale production of recombinant protein in *P. pastoris* was done essentially as described by Stratton et al., 1999 and as detailed by Silva et al., 2011, except that the temperature for the Glycerol Batch and Glycerol Fed-Batch phases was 30 °C and then adjusted to 25 °C for the Methanol Fed-Batch phase in order to improve the stability of the enzyme. Agitation was specifically controlled below 750 rpm to avoid excessive cell debris from the *P. pastoris* cells and to limit the downstream purification process. Additional oxygen was added automatically to accommodate optimal growth and enzyme expression.

At the end of the fermentation process the *Pichia* cells were collected by centrifugation at 5300 *g*, 5°C for 1 hour. The supernatant was subjected to sterile filtration, followed by concentration by ultrafiltration using a cross-flow bioreactor system. The enzyme preparations containing 25% (w/v) glycerol were stored at -80°C.

The purification was performed by Cu^{2+} affinity column chromatography using a CIM® IDA-8f ml Tube Monolithic Column purchased from BIA Separations GmbH (Villach, Austria). The affinity chromatography was carried out as described by Silva et al, 2011. The imidazole was removed using PD-10 columns (GE Healthcare).

DETERMINATION OF THE OPTIMAL CONDITIONS OF THE ENZYMATIC REACTIONS

MODDE Version 7.0.0.1 (Umetrics AB, Umeå, Sweden) was used as a tool to design the experimental frame and to fit and analyze the data. The factors influencing the reactions yields were tested: pH, temperature - for all the investigated enzymes (endo-1,4-β-galactanase from *Emericella nidulans*; *Trypanosoma rangeli* mutant sialidase with 6 point mutations, Tr6; *Trypanosoma rangeli* mutant sialidase from Tr6 parent with 7 point mutations, Tr13). Moreover, enzyme to substrate ratio E/S (v/w) and reaction time were tested for endo-1,4-β-galactanase from *E. nidulans´*, and acceptor concentration was investigated for Tr6 and Tr13. The donor substrate, casein glycomacropeptide (cGMP) for Tr6 and Tr13 was kept at constant concentration. The yield of the product was determined by reducing sugar assay in case of optimization of reaction conditions for endo-1,4-β-galactanase from *E. nidulans* and by HPAEC or LC/MS of the products in case of optimization of reaction conditions for Tr6 and Tr13.

ENZYMATIC REACTIONS

The enzymatic reactions were done in conditions determined as the best for the characterized enzymes: endo-1,4-β-galactanase from *E. nidulans*, Tr6 and Tr13. The products were then fractionated by filtration – in case of degradation of galactan or solubilised potato pulp polysaccharides (SPPP), or purified by anion exchange chromatography in case of synthesis of human milk oligosaccharides (HMOs) and human milk- like oligosaccharides.

ANALYTICAL METHODS

High- performance size exclusion chromatography (HPSEC) was applied to determine progress of enzymatic degradation of potato galactan and SPPP.

High-performance anion exchange chromatography with pulsed amperometric detection was used to monitor profile of oligosaccharides released from potato galactan (Megazyme) by the endo-1,4-β-galactanase from *E. nidulans*; to determine concentration of monosaccharides obtained by acid hydrolysis of complex saccharides; to determine concentration of 3'-sialyllactose, a model human milk oligosaccharide, and sialic acid which were produced by both Tr6 and Tr13; to detect release of arabinose and galacturonic acid from modified SPPP samples treated with arabinofuranosidase and exo-polygalacturonase, respectively.

Acid hydrolysis of the galactan and SPPP was done by a modified method described by Garna et al., 2004. At a concentration of 2.5 g/L or 4 g/L each substrate was treated with 0.2 M trifluoroacetic acid for 72 h at 80◦C. The recovery of monosaccharides was determined by performing the same hydrolysis on L-rhamnose, L-arabinose, D-galactose, D-glucose, and D-galacturonic acid.

BACTERIAL GROWTH ASSAYS

The saccharide samples were dissolved in water at 10% (w/v) and sterilized by sterile filtration or by UV-radiation for 30 s. Different bacterial strains were tested including prebiotic, pathogenic and commensal bacteria. These strains were incubated in mono-cultures with the individual substrates at concentrations of 1% (w/v) in multiwell plates in a Bioscreen® C system (Labsystems, Helsinki, Finland) as described by Mäkeläinen et al., 2010. The bacterial growth was determined as a function of OD_{600} and time. The baseline growth in the media without addition of carbohydrates was used as a control and subtracted from growth obtained in the presence of substrate. Fructooligosaccharides (Raftilose®, Beneo, Belgium) or galactan (Megazyme International LTD) were used as an established prebiotic standard.

METHODS SPECIFIC FOR CHARACTERIZATION OF ENDO-1,4-β-GALACTANASE FROM *E. NIDULANS* EXPRESSED IN *P. PASTORIS*

Thermostability investigation

The thermal stability was evaluated by preincubation of the enzyme in 5 mM NaH_2PO_4 buffer (pH 5) at 50, 60 and 70 ◦C for 2–60 min. After a set exposure time at the selected temperature, an aliquot of the enzyme solution was cooled to 50 ◦C, galactan solution was added and the residual enzyme activity was measured via the reducing sugar assay.

Influence of metal ions on enzyme activity

The enzyme was incubated with 10 mM EDTA for 40 min at room temperature. The control sample was incubated with 5 mM phosphate buffer pH 5 instead of the EDTA solution. After incubation with EDTA, the EDTA was removed by gel filtration (PD-10 columns, GE Healthcare). The relevant metal ion solution was added (1 mM) to aliquots of EDTA pretreated and control samples, respectively (no metal addition was a benchmark both in the EDTA pretreated and in the control samples). The reducing sugar assay was used to determine the residual enzyme activity.

METHODS SPECIFIC FOR MODIFICATION OF SPPP

The SPPP was at first treated with an α-amylase to remove the residual starch. Subsequently it was deactetylated and demethylated by use of NaOH solution. This was followed by treatment with RGI lyase to degrade the remaining RGI backbone and make it more available for other enzymes. Obtained material was divided in four fractions, which were enzymatically modified in four different ways. The first fraction was treated with endo-1,4-β-galactanase; the second with α- L- arabinofuranosidase and subsequently with endo-1,4-β-galactanase; the third one with exo-polygalacturonase and subsequently with endo-1,4-β-galactanase; the fourth one with exo-polygalacturonase, then with α- L- arabinofuranosidase and eventually with endo-1,4-β-galactanase. The monomers that were released during incubation with arabinofuranosidase and exo-polygalacturonase were removed by dialysis. The chain of chemical and enzymatic modifications that the SPPP was subjected to is shown in Figure 17 in chapter 7.

Removal of residual starch

The SPPP was treated with an α-amylase from *Bacillus licheniformis* to remove the residual starch present in the material. The reaction was performed in 50 mM sodium dihydrogen phosphate buffer pH 6, at 70°C for 85 min. The substrate concentration was 6 g/L and the enzyme to substrate ratio (E/S) was 0.2% (v/w). The reaction was stopped by boiling for 10 minutes. The post-reaction mixture was then dialyzed against deionized water using a cross-flow Hydrosart membrane system with 2 kDa cut-off (Sartorius AG, Goettingen, Germany). The permeate containing glucooligosaccharides was discarded.

Deesterification

The retentate obtained in starch removal process was subjected to treatment with 40 mM sodium hydroxide as described by Holck et al., 2011. The mixture was then neutralized by use of 1 M HCl. Subsequently, filtration with a cross-flow Hydrosart membrane system with 2 kDa cut-off was performed to remove salts and the reaction product was freeze-dried.

RGI lyase treatment

The obtained material was incubated with RGI lyase from *B. licheniformis*. Reaction was performed for 1 h at 60°C in 10 mM EPPS buffer at pH 8. The reaction mixture comprised of 5 g/L of SPPP and enzyme to substrate ratio (w/w) of 2.3%. To enhance the enzyme activity 2 mM solution of $MnCl_2$ was applied in the reaction mixture, as described by Silva et al., 2011. To stop the reaction the enzyme was heat-inactivated. The post-reaction mixture was dialyzed against deionized water using the same cross-flow system as described above, and the manganese ions and EPPS buffer were removed from SPPP solution. The modified SPPP was lyophilized.

α- L- Arabinofuranosidase treatment

The incubation of SPPP material with α- L- arabinofuranosidase from *E. nidulans* was performed at substrate concentration of 6 g/L and enzyme to substrate ratio (v/w) of 0.5%. The reaction was allowed to proceed for 2 h at 60°C and pH 5. The pH was adjusted with 0.5 M HCl or 0.5 M NaOH (if the reaction was done after incubation with exo-polygalacturonase). After heat-inactivation of the enzyme, the post-reaction mixture was dialyzed to remove arabinose. The retentate - product was freeze-dried.

Exo-polygalacturonase treatment

SPPP was incubated with exo-polygalacturonase from *E. nidulans* at 6 g/L of substrate concentration and enzyme to substrate ratio (w/w) of 1% for 2 h. The reaction conditions were 40ºC and pH 4, which was adjusted with 0.5 M HCl. After heat-inactivation of the enzyme, the post-reaction mixture was dialyzed to remove galacturonic acid. The obtained product was lyophilized.

Endo-1,4-β-galactanase treatment

The incubation of SPPP with endo-1,4-β-galactanase from *E. nidulans* was done at substrate concentration of 6 g/L and enzyme to substrate ratio (v/w) of 0.3%. The reaction was allowed to proceed for 15 min at 50°C and pH 5. The pH was adjusted with 0.5 M HCl and 0.5 M NaOH. After inactivation of the enzyme, the product was freeze-dried

4 ENZYMATIC SYNTHESIS OF HUMAN MILK OLIGOSACCHARIDES

This chapter is based on the publication "Biocatalytic production of 3'-sialyllactose by use of a modified sialidase with superior trans-sialidase activity" enclosed in chapter 10.1.

4.1 SPECIFIC HYPOTHESES AND OBJECTIVES

Hypotheses for synthesis of human milk oligosaccharides:

- It is possible to tune a mutated sialidase from *Trypanosoma rangeli* (Tr6) to trans-sialidase by optimizing the reaction conditions.
- Tr6 has a potential to catalyse synthesis of human milk oligosaccharides (HMOs) from casein glycomacropeptide (cGMP) and oligosaccharides.

These hypotheses were verified by conducting the following activities:

- Optimization of reaction conditions for mutated sialidase from *T. rangeli*, Tr6 to promote its trans-sialidase activity.
- Synthesis of human milk oligosaccharides catalysed by Tr6.

4.2 RESULTS AND DISCUSSION

An enzymatic synthesis of HMOs can be achieved by a trans-sialidase from *T. cruzi*, the causative agent of Chagas disease. However, for industrial production of food-grade HMOs, it is a drawback that the enzyme constitutes an important virulence factor within *T. cruzi*. Producing mutants of the non-pathogenic *T. rangeli* sialidase which possesses some trans-sialidase activity (Paris et al., 2005) is an attractive alternative for application in production of functional food.

4.2.1 Production and characterization of mutant sialidase Tr6

The enzyme produced for the purpose of generating HMOs was a mutant sialidase from *T. rangeli*, Tr6, which contained following mutations: M96V, A98P, S120Y, G249Y, Q284P and I37L (Paris et al., 2005). It was expressed in *P. pastoris* after codon-optimization. The Tr6 contained six point mutations and included C-terminal c-myc and 6xHis tag. A positive transformant expressing the Tr6 enzyme was selected and used for enzyme production at a 5 L-scale. The enzyme concentration reached 1 g/L at the end of fermentation. This may be considered as a high concentration, since the concentration of produced previously *T. cruzi* trans-sialidase reached only 5 mg/L (Sabesan and Paulson, 1986). The relatively high yield of Tr6 production may be ascribed to the codon

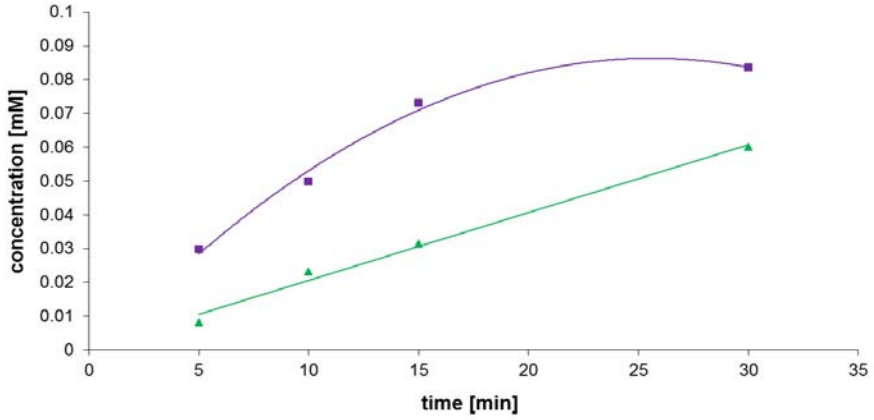
optimization or to the more controlled fermentation conditions and higher cell density achieved in the fermentor than in the shaking flasks.

The produced enzyme had an apparent molecular weight of about 80 kDa by SDS-PAGE. The treatment of the Tr6 with EndoH followed by Western blotting analysis revealed that the expressed protein was glycosylated and the molecular weight of Tr6 was 75 kDa after deglycosylation.

The Tr6 mutant enzyme showed a high level of sialidase activity and a relatively low level of trans-sialidase activity (Paris et al., 2005). In order to change the ratio between hydrolytic and trans-sialidase activities, the reaction conditions were investigated. pH, temperature, donor and acceptor concentrations optimal for trans-sialylation were determined in statistically designed experiments. The optimization of reaction conditions was done for a model reaction of synthesis of HMOs, i.e. for production of 3'-sialyllactose. Casein glycomacropeptide (cGMP) and lactose, side-streams from dairy industry, were used as substrates in synthesis of 3'-sialyllactose. Promotion of trans-sialidase activity occurred at 25 °C and pH of 5.5. However, in the reaction conducted at these conditions and with 36.8 mM lactose and 2.6 mM sialic acid bound in cGMP the concentration of sialyllactose was relatively low and the exhibited hydrolytic activity was still significant (Figure 5A). Therefore, higher concentrations of acceptor were tested, since the high acceptor: donor ratios are known for promoting trans-glycosylation reactions. The increase in lactose concentration led to the production of high concentration of 3'-sialyllactose by Tr6 (Figure 5B). The established conditions: pH 5.5., 25°C, 4.6 mM sialic acid bound in cGMP (equivalent to 26 g/L of cGMP) and 117 mM lactose (40 g/L) tuned the mutated sialidase from *T. rangeli,* Tr6 into an effective sialyl-transferase with more than 93% trans-sialidase activity and only 7% residual sialidase activity.

At the high acceptor concentration of 117mM the specific trans-sialidase activity of the Tr6 was determined to be 16.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. The specific activity was thus ~4 times higher than the reported V_{max} of the *T. cruzi* trans-sialidase which was 3.8 $nmol \cdot min^{-1} \cdot \mu g^{-1}$ measured using 3'-sialyllactose as the donor and lactose as the acceptor (Paris et al., 2005). It was also ~450 times higher than the rate at which trans-sialidase from *T. cruzi* catalysed synthesis of sialyllactose when 5 g/L cGMP and 20 g/L lactose were used as substrates (Pelletier et al., 2001). A sialidase from *Bifidobacterium infantis* catalysed trans-sialylation, employing high levels of both cGMP and lactose (McJarrow et al., 2003), and produced only low level of sialyllactose at a rate which was 87-fold lower than that obtained with Tr6.

A)



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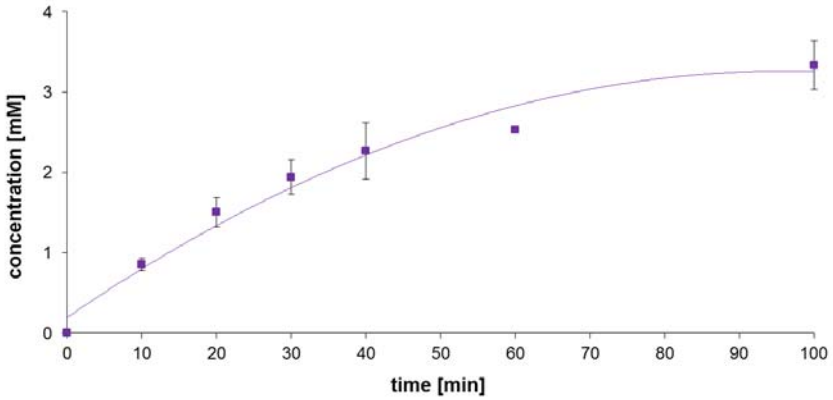


Figure 5. A) Accumulation of 3'-sialyllactose and sialic acid over time (25 min) at low concentration of lactose: 36.8 mM lactose, at 25°C, pH 5.5, and 2.6 mM sialic acid contained in cGMP; measured by HPAEC-PAD; 3'-sialyllactose is shown in violet and sialic acid in green. B) Accumulation of 3'-sialyllactose over time at high lactose concentration, 117 mM lactose, 25°C, pH 5.5, and 4.6 mM sialic acid bound in cGMP; measured by LC/MS. No free sialic acid was detected.

4.2.2 Production of HMOs

When the reaction conditions promoting transfer of sialic acid were established, the synthesis of 3'-sialyllactose was performed in a 5L scale. 5.3 mg/L of Tr6 was incubated for 20 min at pH 5.5, 25 °C, with 117 mM lactose and 4.6 mM sialic acid residues bound in cGMP. After heat-inactivation of the enzyme, the product was purified in two steps: at first by ultrafiltration to remove cGMP and Tr6, which was followed by freeze-drying, and then by anion exchange chromatography using ammonium formate as the eluent (Figure 6).

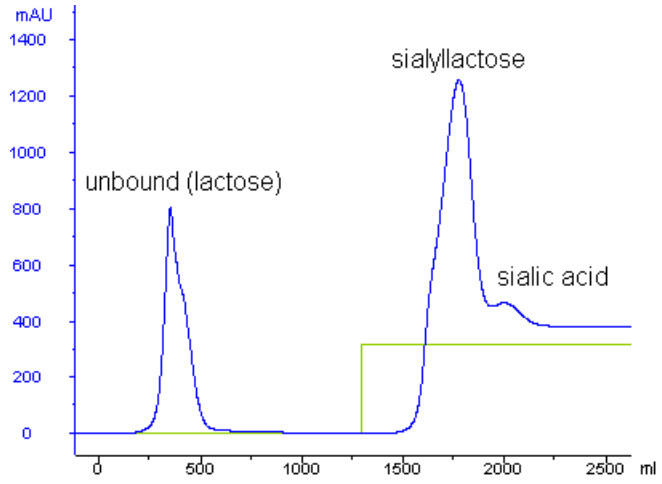
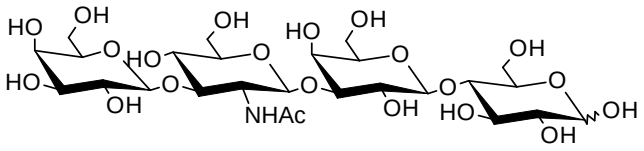


Figure 6. Purification of 3'-sialyllactose by anion exchange chromatography. The green line indicates the elution gradient with 40 mM ammonium formate.

The high quality of the separation of 3'-sialyllactose from sialic acid and lactose, as well as the identity of this compound was confirmed by capillary LC/MS and NMR spectroscopy. The removal of ammonium formate was, however, troublesome. The samples had to be lyophilized six times to remove the buffer and achieve its content in the product below 5%. The final yield of 3'-sialyllactose was 3.6 g, and the amount of free sialic acid was 133 mg. About 50% of the sialic acid residues in the cGMP are bound at the 3'- position, whereas the rest is bound at the 6'-position (Saito and Itoh, 1992). Since Tr6 catalysed only the production of 3'-sialyllactose, it can be assumed that only 3'-bound sialic acid, i.e. about 50% of the total sialic acid in cGMP, was available for this enzyme. Therefore, the molar yield of 3'-sialyllactose was 47.7% based on the available 3'-sialic acid in cGMP. The molar yield of the 3'-sialyllactose was 1% on the lactose, and the yield of free sialic acid was 2% based on the total sialyl-residues in the cGMP.

The Tr6 was also able to catalyse the transfer of sialic acid moiety to lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaose I (LNFP I) and lacto-*N*-fucopentaose V (LNFP V) (their structures are shown in Figure 7). The trans-sialidation of these compounds was done at much smaller scale that the 3'-sialyllactose production due to the limited amount of the acceptors. The yields of these products were lower than the yield of 3'-sialyllactose. 5-12 mg of sialylated LNT, LNnT, LNFP I and LNFP V were produced at pH 5.5, 25 °C and as high as possible acceptor/donor ratio.

a)



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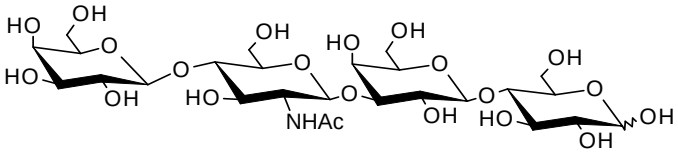

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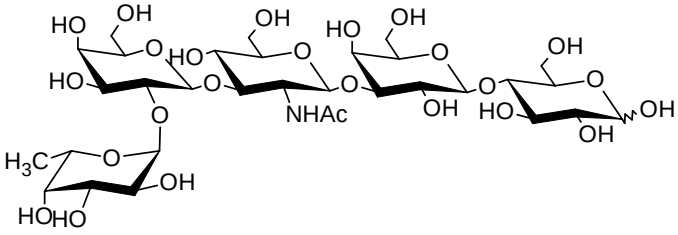

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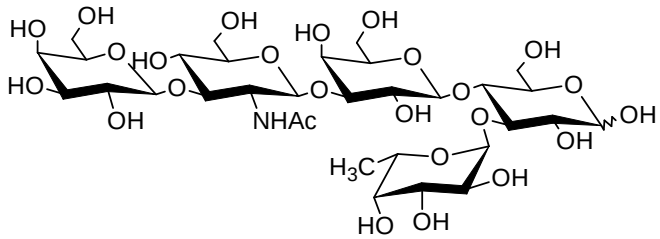

Figure 7. The structures of sialylated oligosaccharides; a) LNT; b) LNnT; c) LNFP I; d) LNFP V.

4.3 CONCLUSION

The mutant sialidase from *T. rangeli*, Tr6 was tuned into an effective trans-sialidase by manipulating the reaction conditions. At the improved conditions the enzyme synthesized 3'-sialyllactose from lactose and cGMP donating sialic acid. Other sialylated HMOs molecules were also produced by this enzyme, i.e. lacto-*N*-tetraoses and lacto-*N*-fucopentaoses. The versatility of the Tr6 trans-sialidase for generating different HMOs was therefore demonstrated. The possibility to create a value-added product from side-streams was demonstrated too. This constitutes an asset for developing a competitive process for novel functional food ingredients.

5 ENZYMATIC SYNTHESIS OF HUMAN MILK-LIKE OLIGOSACCHARIDES

This chapter is founded on the publication "Rational design of a new *Trypanosoma rangeli* trans-sialidase for efficient sialylation of glycans" enclosed in chapter 10.2.

5.1 SPECIFIC HYPOTHESES AND OBJECTIVES

The hypotheses were following:

- It is possible to diminish hydrolytic activity or increase trans-sialidase activity of *T. rangeli* mutant sialidase Tr6 by introducing further mutations.
- It is possible to synthesize human milk and human milk-like oligosaccharides by use of a new mutant sialidase, Tr13.
- Human milk-like oligosaccharides exhibit similar biological properties as these of HMOs, including prebiotic activity.

The aims were:

- To compare hydrolytic and trans-sialidase activities of parent clone Tr6 and the derived new mutants.
- To determine reaction conditions for trans-sialylation catalysed by the best mutant, Tr13.
- To use Tr13 to catalyse synthesis of human milk-like oligosaccharides.
- To investigate prebiotic properties of sialylated glycans produced by means of Tr13.

5.2 RESULTS AND DISCUSSION

5.2.1 Production and characterization of trans-sialidase Tr13

In order to identify mutations likely to affect enzyme activity, amino acids within 14 Å of the sialic acid-binding site were investigated. Basing on an alignment between mutated *T. rangeli* sialidase Tr6 and the efficient trans-sialidase of *T. cruzi*, the chemical difference between amino acids, assuming that larger chemical differences, correlating with lower probability of occurrence by random evolution, were the most likely candidates for conferring increased trans-sialidase activity and/or reduce unwanted hydrolysis. Seven amino acids 197-203 at the border of substrate binding cleft were substituted in Tr6 from IADMGGR to VTNKKKQ to achieve the same amino acids as in *T. cruzi* trans-sialidase (TcTS). The new motif introduced in Tr6 had a net charge change from neutral to +3. The new enzyme was called Tr13. A homology model of mutant Tr13 based on Tr6 with the additional seven mutations is shown in

Figure 8. The mutations are relatively far, ~14 Å, from the acceptor binding cleft and therefore unlikely to affect acceptor binding directly. The highly unusual +3 charge and increase of hydrogen bond donation implies that the water network may be inverted. This could change the electrostatic field in the cleft, and potentially disrupt or even reverse the water network in the active site, which could result in decreased hydrolytic activity of the enzyme.

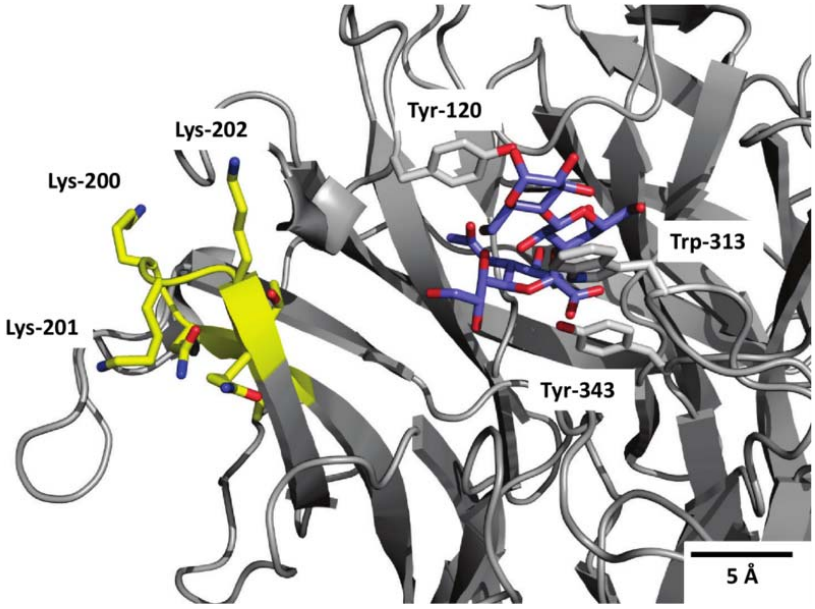

Figure 8. Close-up of active site with sialyllactose docked (blue). Acceptor binding site residues Tyr-120 and Trp-313, and catalytic nucleophile Tyr-343 side chains are shown in grey. The seven introduced amino acids are shown in yellow.

Besides the seven-amino acids motif introduced in Tr13, several single mutations were made from Tr6. The mutations were chosen because of their positions close to the binding cleft and/or by their chemical difference. The mutants were produced in *P. pastoris* shaking flasks cultures and their trans-sialidase activity was tested. Only Tr13 and Tr6 D363E performed at a level comparable to Tr6 while all other mutants displayed a decreased trans-sialidase activity.

Mutants Tr13 and Tr6 D363E were then produced in 5L scale. This enabled further evaluation of their hydrolytic and trans-sialidase activities (Figure 9). Hydrolase activity was tested on *para*-nitrophenyl neuraminic acid (pNP-NeuAc), cGMP, 3'-sialyllactose and 6'-sialyllactose. None of the enzymes: Tr6, Tr6 D363E and Tr13 was active on 6'-sialyllactose. Therefore it was unlikely that the α-2,6-linked sialic acid in cGMP was used by these enzymes in trans-sialylation reaction. The α-2,6-linked sialic acid constitutes about 50 % of total sialic acid content in cGMP (Saito and Itoh, 1992).

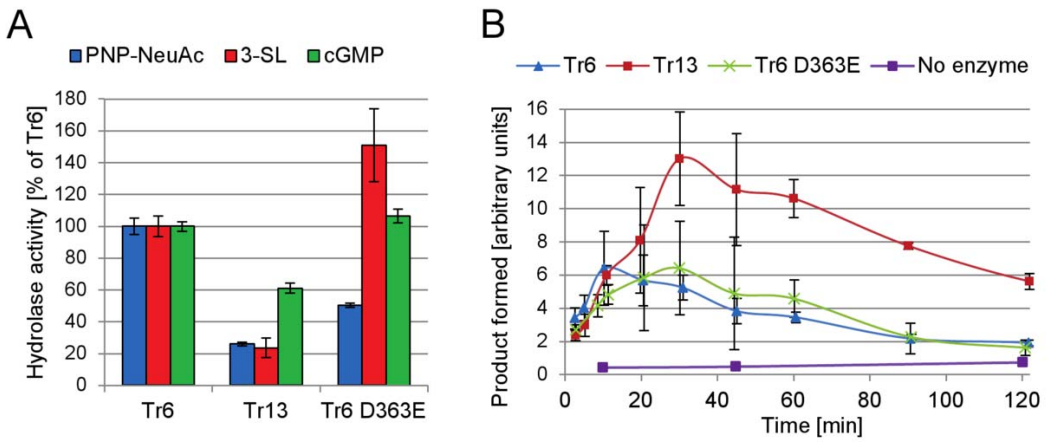

Figure 9. Enzyme activity of Tr6 and selected mutants Tr13 and Tr6 D363E. A) Hydrolase activity on substrates pNP-NeuAc, 3'-sialyllactose (3-SL), and cGMP. B) Trans-sialidase activity using cGMP as sialic acid donor and 4-methylumbelliferyl-β-D-galactopyranoside (MU-Gal) as acceptor.

Tr13 had lower hydrolytic activity than Tr6 on *p*-nitrophenyl neuraminic acid, cGMP and 3'-sialyllactose. Tr6 D363E had hydrolytic activity lower than Tr6 only on the artificial substrate pNP-NeuAc (Figure 9 A). Figure 9 B shows results of trans-sialidase activity assay. The initial reaction rate represents the trans-sialidase reaction. The maximal product concentration represents both trans-sialidase and hydrolase activity, since the product is created and hydrolysed at the same time by the enzyme. As can be seen, Tr13 and Tr6 have the same transferase activity, but Tr13 has lower hydrolytic activity and therefore more product can be accumulated in the reaction mixture. The product concentration is two times higher in case of Tr13 than Tr6. This can be attributed to the seven-amino acids motif introduced into Tr13 structure. The improved product yield, in case of Tr13, suggests that this motif does not influence acceptor binding affinity, but reduces the hydrolytic activity. Tr13 is then hydrolysis-impaired sialidase mutant.

Determination of reaction conditions favouring the trans-sialidase activity of Tr13 was performed by statistically designed experiments. Temperature (20-60 °C), pH (3-5) and acceptor – lactose concentration (117-351 mM) were investigated, whereas donor-cGMP concentration was kept fixed at 8mM cGMP-bound sialic acid. Reactions were performed for 20 min and stopped by heat-inactivation of the enzyme at 90 °C. The product- 3'-sialyllactose concentration was determined by HPAEC. No product formation was observed in controls with heat-inactivated enzyme. The highest product yield was obtained at 351 mM lactose, pH 3 and 20 ºC. A time study was conducted at these conditions (Figure 10) and the enzyme specific trans-sialidase activity was measured to be 4.4 ± 0.7 nmol*min^{-1} per µg of enzyme. From the time study depicted in Figure 10 it can be seen that the reaction time could be extended to 100 min with beneficial increase of product concentration.

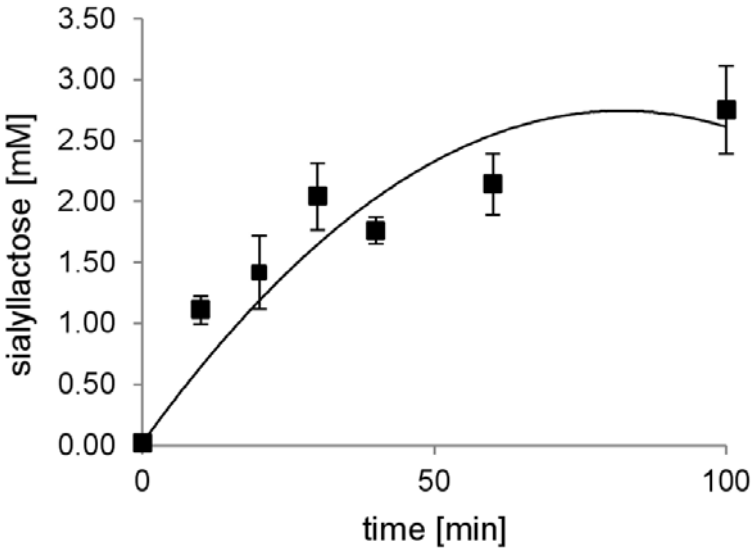

Figure 10. Time study of trans-sialylation catalysed by Tr13. Accumulation of 3'-sialyllactose over time in 20°C, pH 3, 351 mM lactose and 8mM cGMP-bound sialic acid.

5.2.2 Production of human milk- like oligosaccharides

When the reaction conditions for trans-sialidase activity of Tr13 were established, the acceptor specificity of the enzyme was tested by incubating Tr13 with different acceptor molecules. The purpose was to synthesize molecules of similar structures to the human milk oligosaccharides, since they could exhibit similar biological effects and at the same time, they would be cheaper to produce, because production of HMOs other than sialyllactose is very expensive due to the expensive acceptor substrates. The acceptors for production of human milk- like oligosaccharides were some prebiotics, i.e. galactooligosaccharides, iso-maltooligosaccharides and lactulose. Combining the prebiotic effect with sialic acid could result in enhanced beneficial activity. Furthermore, two compounds similar in structure to lactose, i.e. maltose and melibiose were chosen as substrates, because they seemed to be relatively easy to sialylated by the enzyme. One monosaccharide – fucose was used as a substrate as well, since the human milk oligosaccharides contain fucose and sialic acid in their structures. It seemed to be interesting to synthesize and investigate the properties of molecule, which possess both these functional moieties - sialylfucose.

For TcTS it is generally accepted that terminal galactose is an acceptor of sialic acid moiety (Cross and Takle, 1993). By the means of Tr13 it was possible to employ not only terminal galactose, but also terminal glucose, as well as glucose and fucose monomers as attachment sites for sialic acid. The sialylated oligosaccharides were produced at pH 3, ambient temperature, 351 mM sialic acid acceptor and 8 mM cGMP-bound sialic acid by incubation for 20 minutes. After deactivation of the enzyme, the products were purified by ultrafiltration to remove cGMP and enzyme and subsequently by anion exchange chromatography with ammonium formate as eluent (Figure 11). In the purification by chromatography the unbound fraction contained unreacted neutral

acceptor, which was eluted with water. Sialylated products, negatively charged, were eluted with 40 mM ammonium formate. They were followed by free sialic acid, also eluted with 40 mM ammonium formate. According to LC/MS analysis the products were completely separated from sialic acid and their precursor acceptor. The highest yields were achieved when fucose (1.5 mM) and lactulose (1.9 mM) were used as the acceptors (Table 6). The product yields of GOS and IMO were lower. GOS and IMO were mixtures of oligosaccharides of different DP. The product yields for different DP might have been different from the average. There were four sialylated oligosaccharides generated from GOS, including 3'-sialyllactose, and five created from IMO, including sialylglucose (Table 6). Since sialyllactulose was produced in the highest molar yield, it could be speculated that the presence of galactose and the 1,4-β bond between galactose and fructose might make it more easily accessible for Tr13, since the yield of the similar-size acceptors melibiose (1,6-α-bound galactose) and maltose (1,4-α-bound glucose) was more than 40 % lower.

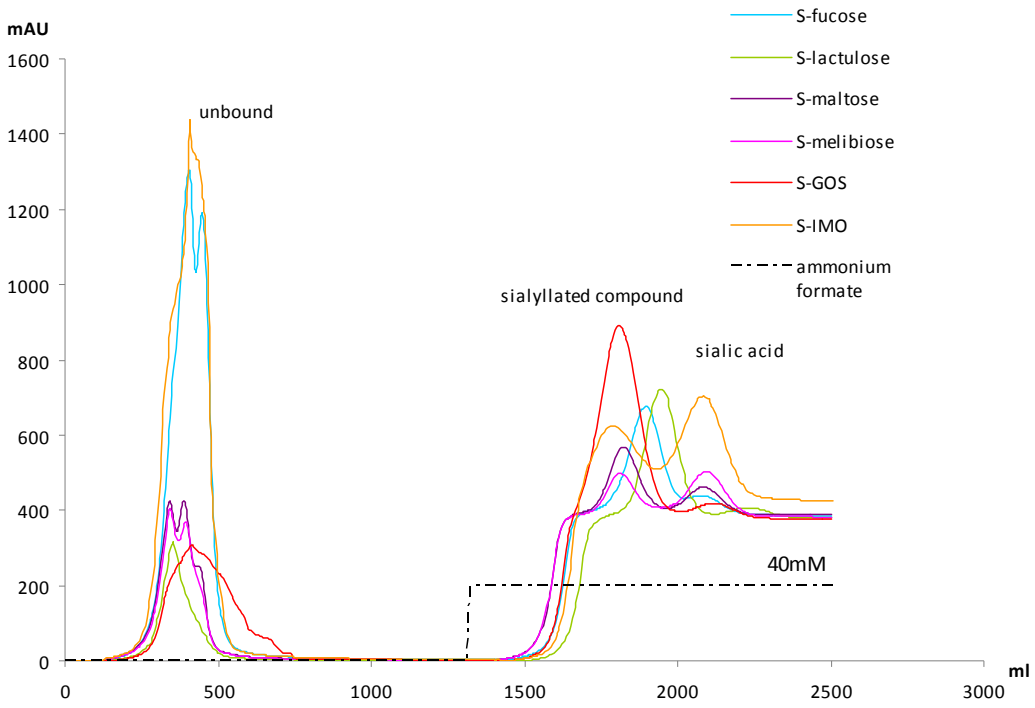

Figure 11. Anion exchange chromatogram of purification of sialylated glycans.

The fact that Tr13 sialylated not only terminal galactose, but also terminal glucose and even glucose and fucose monomers was surprising and indicated a high level of acceptor substrate promiscuity. This broad specificity may have a great potential in the synthesis of many sialylated glycans.

acceptor	m/z	product	product conc.	product yield	
	$[M-H]^-$		[g/L]	[w_{prod}/w_{accep}]	[mM]
GOS	632	SA-α-Gal-1,4-β-Glc	1.0	0.44%	ND
	794	SA-α-Gal-1,4-β-Gal-1,4-β-Glc			
	956	SA-α-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Glc			
	1118	SA-α-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Glc			
Fucose	454	SA-α-Fuc	0.66	1.17%	1.46
Melibiose	632	SA-α-Gal-1,6-α-Glc	0.62	0.52%	0.98
Lactulose	632	SA-α-Gal-1,4-β-Fru	1.2	0.97%	1.84
Maltose	632	SA-α-Glc-1,4-α-Glc	0.66	0.55%	1.04
IMO	470	SA-α-Glc	0.72	0.60%	ND
	632	SA-α-Glc-α-Glc			
	794	SA-α-Glc-α-Glc-α-Glc			
	956	SA-α-Glc-α-Glc-α-Glc-α-Glc			
	1118	SA-α-Glc-α-Glc-α-Glc-α-Glc-α-Glc			

Table 6. Products of sialylation of various glycans analysed by LC/MS. Yields are given as product concentration and as % (w/w) of product produced from acceptor used. ND: the molar concentration of sialylated GOS and IMO could not be calculated since the distribution of different chain lengths was not determined. Abbreviations: SA, sialic acid; Gal, galactose; Glc, glucose; Fuc, fucose; Fru, fructose.

5.2.3 Investigation of prebiotic properties of sialylated glycans

The enzymatically synthesized sialylated glycans were tested in single bacterial cultures towards potential prebiotic properties. Six strains of bifidobacteria were tested: *B. longum longum* (Danisco Global Culture Collection DGCC 232), *B. longum infantis* (DGCC 233), *B. longum infantis* (DGCC 1497), *B. longum infantis* (DGCC 2238), *B. longum* (Bl-05, DGCC 9917), *B. lactis* (HN019, DGCC 2013). *L. acidophilus* (NCFM, ATCC 700396) and *C. perfringens* (ATCC 13124) were tested as well. The experiments were done in a Bioscreen microtiter system, and the bacterial growth was expressed as an area under the growth curve (Mäkeläinen et al., 2010).

The results of bacterial growth on sialylated glycans are presented in Table 7. Sialylated melibiose and maltose did not promote growth of prebiotic bacteria. The growth of *B. infantis* 233, *B. infantis* 1497, and *B. longum* 232 was promoted by different sialylated compounds while sialylated fucose promoted growth of all three. The growth of *B.*

infantis 2238, *B. lactis*, *L. acidophilus*, and *B. longum* 9917 was not promoted by the sialylated compounds. *L. acidophilus,* however, grew well on the prebiotic control-galactan. *L. acidophilus* is known for possessing β-galactosidase which should enable its growth on galactan (Schwab and Gänzle, 2011). Moreover, in the publication by Schwab and Gänzle, 2011, *L. acidophilus* was reported to exhibit only a very weak growth on 3'sialyllactose, whereas it grew well on 6'-sialyllactose. This could explain why it did not grow on other oligosaccharides containing 3'-sialyl group. *C. perfringens* grew significantly better than all the probiotic strains on the sialylated compounds, except sialylmelibiose, on which the growth was not investigated due to the lack of material.

Bacterial strain	Area under the growth curve [OD_{600} x min]							
	MRS-	S-GOS	S-fucose	S-melibiose	S-lactulose	S-maltose	S-IMO	galactan
B. infantis 233	30 ± 14	132 ± 5	71 ± 6	14 ± 6	109 ± 20	30± 4	95 ± 23	55 ± 7
B. infantis 2238	294 ± 68	274± 18	285± 15	302 ± 2	269± 20	158± 24	269± 6	264± 7
B. infantis 1497	31± 10	42 ± 13	149 ± 2	ND	34± 2	ND	45± 1	40± 9
B. longum 232	79± 20	162± 9	192 ± 20	104± 17	134± 19	122± 31	107± 19	42±13
B. lactis	139± 70	176 ± 18	192± 27	ND	122±18	102± 8	175± 13	143± 15
L. acidophilus	180± 28	159± 4	188± 18	192 ± 4	128± 2	193± 12	217± 19	371± 10
B. longum 9917	106± 30	71± 5	114± 15	70± 8	34± 14	101± 6	103± 9	93± 44
C. perfringens	455± 32	722± 52	811± 48	ND	541± 17	844 ± 99	1098± 61	447 ± 46

Table 7. Bacterial growth on sialylated glycans is given as an area under the growth. Growth responses for the substrates are shown for a substrate concentration of 10 g/L for all bacterial strains. Data are given as average values of 3 replicates ± s.d. The growth of *B. longum infantis* 1497, *B. lactis* and *C. perfringens* was not tested on sialylmelibiose, as well as growth of *B. longum infantis* 1497 on sialylmaltose (ND). Galactan was used as a control.

Despite variations in growth, some of the probiotic bacteria were able to utilize sialylated saccharides. The sialylated compounds did not, however, stimulate selectively their growth. A prerequisite for utilizing the sialylated compounds should be the presence of a sialidase and/or the ability to degrade the prebiotic backbone. All the *B. longum* subsp. *infantis* strains contain a sialidase and *C. perfringens* also contains the necessary enzymes for metabolising sialic acid (Walters et al., 1999). Therefore, a mixed culture experiment that would reflect the conditions in the human gastrointestinal tract, e.g. cross-feeding effect between bacteria or release of short chain fatty acids and their influence on different strains, especially pathogenic, is required to assess the prebiotic properties of human milk-like oligosaccharides. In the publication by Yu et al., 2013 three fucosylated HMOs were shown to stimulate bifidobacteria, while *E. coli* and *C. perfringens* were unable to utilize these compounds. The fermentation products, i.e.

SCFA inhibited the growth of pathogens. It might be speculated that this could also take place in a mixed cultures when sialylated glycans will be used.

5.3 CONCLUSION

Hydrolytic activity of sialidase from *T. rangeli*, Tr6 was decreased by introducing mutations on the border of substrate binding site. By the means of the new mutated sialidase, Tr13 it was possible not only to produce a model human milk oligosaccharide – sialyllactose, but also other sialylated glycans – human milk-like. The generated sialyloligosaccharides have a significant potential as functional food ingredients. Their production is expected to be cheaper than production of HMOs, since the acceptors used to obtain human milk- like oligosaccharides were much cheaper that these applied in HMOs production (with lactose being an exception). The presence of sialyl moiety in their structures raises expectations that they may act as pathogen decoy molecules and immune system modulators, as HMOs do. Moreover, they are presumed to possess prebiotic properties. The fact that they did not reveal distinguished bifidogenic properties in the single cultures experiments is disappointing, but can be reasonable explained by not including bacterial interactions in these experiments. Therefore further prebiotic tests with mixed bacterial cultures are required to evaluate the prebiotic properties of sialylated glycans.

6 ENZYMATIC DEPOLYMERIZATION OF POTATO POLYSACCHARIDES

This chapter is based on the publication "Expression and characterization of an endo-1,4-ß-galactanase from *Emericella nidulans* in *Pichia pastoris* for enzymatic design of potentially prebiotic oligosaccharides from potato galactans" enclosed in chapter 10.3., and on publication "The binding of zinc ions to *Emericella nidulans* endo-ß-1,4-galactanase is essential for crystal formation" enclosed in chapter 10.4.

6.1 SPECIFIC HYPOTHESES AND OBJECTIVES

The hypotheses on enzymatic depolymerization of galactan and the so-called soluble potato pulp polysaccharides (SPPP) were as follows:

- It is possible to produce galactopoly- and oligosaccharides from pure galactan and SPPP.
- Endo-1,4-ß-galactanase from *Emericella nidulans* can be produced in *P. pastoris* in high amount and purified efficiently.
- Both galactan and SPPP are the right substrates for endo-1,4-ß-galactanase from *E. nidulans*.
- Products of enzymatic hydrolysis of SPPP and galactan possess prebiotic activity.

Verification of these hypotheses was done by:

- Production and purification of endo-1,4-ß-galactanase from *E. nidulans.*
- Establishing the optimal working conditions of endo-1,4-ß-galactanase from *E. nidulans* using galactan as substrate.
- Production and fractionation of galactose rich poly- and oligosaccharides by degradation of galactan and SPPP with endo-1,4-ß-galactanase.
- Determination of prebiotic potential of SPPP and galactan-derived products.

6.2 RESULTS AND DISCUSSION

6.2.1 Production and characterization of endo-1,4-β-galactanase

As mentioned above, the objective of this study was to use potato β-1,4-galactan and the SPPP as substrates for enzymatic production of potentially prebiotic compounds of lower and narrower molecular weight. To achieve that an endo-1,4-β-galactanase from *Emericella nidulans*, GH family 53, was produced in a recombinant *Pichia pastoris* strain. The endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was produced in 5L fermentor. The level of extracellular protein in the fermentation reached

2.11 g/L after 95 h. The endo-1,4- β-galactanase activity was 109 kU/L. After the fermentation centrifugation, sterile filtration and ultrafiltration was performed. The concentrated protein solution was purified by affinity chromatography. The imidazole buffer used for elution of the enzyme from the column was removed by gel filtration. The yield of endo-1,4-β-galactanase in fermentation was 1.6 g/L. This level of heterologously expressed protein may be considered as high (Macauley-Patrick et al., 2005).

The optimal reaction conditions of *E. nidulans* endo-1,4- β-galactanase were determined in statistically designed experiments, in which potato galactan bought from Megazyme was used a substrate. The following factors were investigated: pH, temperature, reaction time and enzyme to substrate ratio, E/S (v/w). The optimal pH and temperature were determined to 5 and 49 ◦C, respectively (Figure 12).

The kinetic parameters K_M and v_{max} for the endo-1,4-β-galactanase was determined to be 0.51 g/L and 73.3 U/mg of protein, respectively, using a Hanes plot. The investigation of the thermal stability showed that the enzyme was stabilized by the presence of the substrate. Without the substrate, the endo-1,4-β-galactanase had a half-life time, t½ of 57 min at 40 ◦C, 27 min at 49 ◦C and 10 min at 60 ◦C.

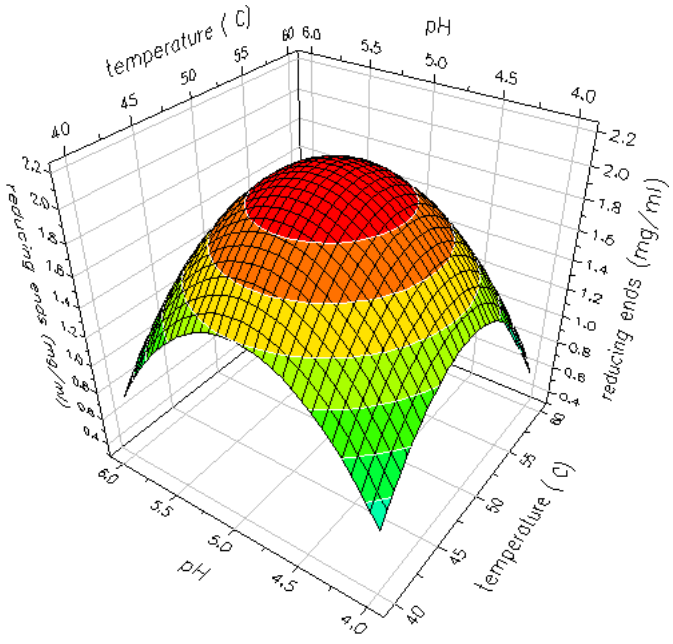

Figure 12. Surface response of the endo-1,4-β-galactanase activity as a function of temperature and pH. The colours vary from blue (low endo-1,4-β-galactanase activity) to red (high endo-1,4-β-galactanase activity).

The influence of metal ions on the enzyme activity was tested, too. The enzyme was pretreated with EDTA prior to incubation with the metal ions. The activity was not affected much by the metal ions. The strongest negative influence had Al^{3+}, whereas the strongest positive effect was achieved by incubation with Zn^{2+} (Table 8). A minor increase in the enzyme activity occurred also in samples incubated with Ca^{2+} and Mn^{2+}.

The literature describes the influence of metal ions on fungal galactanases as weak, but inhibitory. For example, Zn^{2+} and Ag^{+} decrease activity of endo-β-1,4-galactanases from *Aspergillus niger* and *Aspergillus aculeatus* (van de Vis et al., 1991). The explanation for improvement of activity of endo-β-1,4-galactanase from *E. nidulans* expressed in *P. pastoris* in the presence of divalent metal ions, especially Zn^{2+}, was found out during crystallization of the enzyme.

pre-treatment	metal added	activity (%)
none	None	100 ± 3.6
none	$AlCl_3$	73.3 ± 6.4
none	$MgCl_2$	92.7 ± 3.3
none	$MnCl_2$	89.2 ± 1.9
none	$ZnCl_2$	105.7 ± 4.9
none	$CaCl_2$	98.7 ± 5.2
EDTA	None	87.7 ± 1.2
EDTA	$AlCl_3$	51.3 ± 3.3
EDTA	$MgCl_2$	90.8 ± 4.9
EDTA	$MnCl_2$	104.4 ± 0.3
EDTA	$ZnCl_2$	107.5 ± 2.2
EDTA	$CaCl_2$	103.5 ± 2.7

Table 8. Influence of metal ions on the endo-1,4-β-galactanase activity.

For the purpose of crystallization the endo-β-1,4-galactanase was purified by chromatography, as described above. The enzyme solution was concentrated and higher molecular weight aggregates of protein were removed by centrifugation. The crystallization trials were carried out at room temperature. After five months a box-shaped single crystal appeared in only one crystallization mixture containing zinc acetate. The structure of endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was determined by the molecular replacement method using endo-1,4-β-galactanase from *A. aculeatus* (having 72.5% sequence identity) as a search model. The endo-1,4-β-galactanase from *A. aculeatus* crystallized in the presence of calcium ions revealed only one calcium ion located in its structure. One calcium ion was also found in the endo-1,4-β-galactanase from *B. licheniformis*, the least similar to *E. nidulans* enzyme. The structure of *E. nidulans* endo-1,4-β-galactanase showed 15 zinc ions bound in its structure. Figure 13 shows an overlay of endo-1,4-β-galactanase from *E. nidulans* with the closest galactanase from *A. aculeatus* and the least similar bacterial galactanase from *B. licheniformis*.

The fact that the galactanase from *E. nidulans* acted as a "zinc sponge" was surprising, especially in the context of the structures of other galactanases. Fourteen of fifteen zinc ions bound to the structure of *E. nidulans* endo-1,4-β-galactanase were bound to the surface of the enzyme. Furthermore, about half of the zinc ions bridged the enzyme molecules in the unit cells. This phenomenon explains why zinc ions were indispensable for crystallization of the enzyme. In the literature there are known examples of proteins with metal ions from the crystallization conditions bound to the surface (Fass et al., 1997). Moreover, the 15 zinc ions compensate the 29 negative charges of the *E. nidulans* galactanase polyanion and this also helps in the crystallization process and bridges different enzyme molecules in the unit cell.

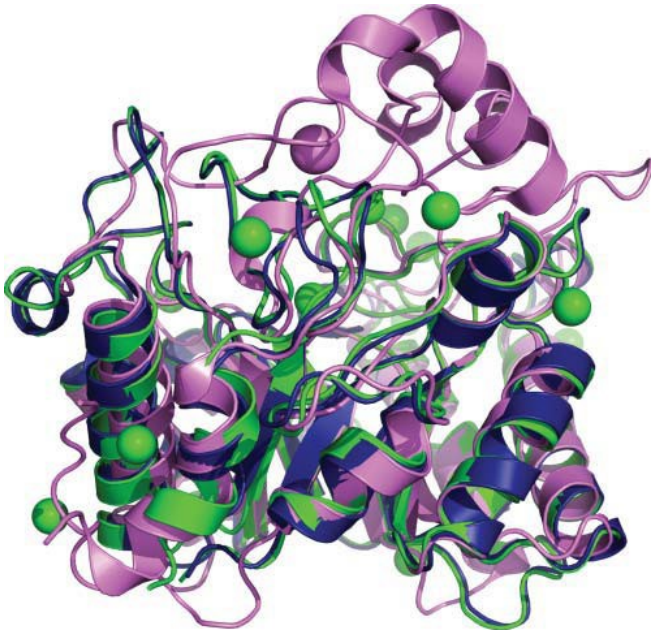

Figure 13. Endo-1,4-β-galactanase from *E. nidulans* (in green) superimposed on endo-1,4-β-galactanases from *A. aculeatus* (in blue) and *B. licheniformis* (in violet). Zinc ions are shown as green spheres and calcium ion in *B. licheniformis* endo-1,4-β-galactanase is shown as violet sphere.

Since the structure did not suggest any catalytic role of the zinc ions, the slightly increased activity of the endo-1,4-β-galactanase in the presence of the zinc ions may be explained by their stabilizing effect on the enzyme structure by neutralization of the surface charge and by better interactions of neutral enzyme with the substrate.

6.2.2 Production of galactosaccharides by means of endo-1,4- β-galactanase

The SPPP, which molecular weight was higher than 400 kDa, were prepared from potato pulp via a short, minimal enzyme treatment with pectin lyase and polygalacturonase, as described by Thomassen et al., 2011. Products of degradation of SPPP with endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* were obtained by treatment of the substrate for 15 min at enzyme to substrate ratio E/S (v/w) ratio of 0.3%. After incubation for 15 min the enzyme was heat-inactivated. The size

exclusion chromatogram of the post-reaction mixture is shown in Figure 14. This mixture was separated by filtration with 10 kDa membrane and two fractions were obtained, the permeate fraction SPPP1 and the retentate fraction SPPP2.

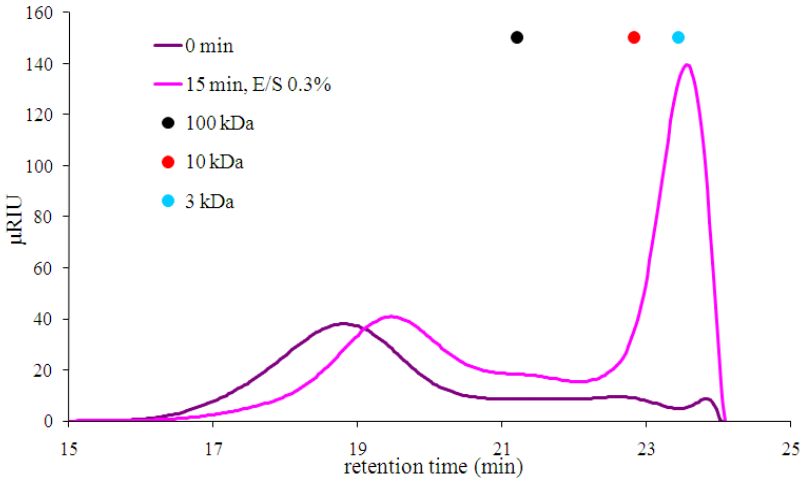

Figure 14. HPSEC analysis of *E. nidulans* endo-1,4-β-galactanase-catalysed hydrolysis of SPPP.

The potato galactan from Megazyme, which had molecular weight of about 150 kDa, was treated with endo-1,4-β- galactanase in the same manner as SPPP. Different reaction conditions were used: 15 min of incubation and E/S (v/w) ratio of 0.3%, 270 min and E/S (v/w) ratio of 0.3%, and 60 min with E/S (v/w) ratio of 0.03% (Figure 15). The obtained post-reaction mixtures were filtered. The mixture produced by incubation for 60 min was separated with 3 and 10 kDa into two fractions: below 3 kDa (fraction PG1; where PG means potato galactan) and between 3 and 10 kDa (PG2). From the substrate incubated for 15 min a fraction below 10 kDa (PG3) and between 10 and 100 kDa (PG4) was produced. From the substrate incubated with enzyme for 270 min a fraction of oligosaccharides below 3 kDa (PG5) was generated

Analysis by HPAEC showed that the products of galactan degradation with endo-1,4-β-galactanase from *E. nidulans* contained free galactose, especially fraction PG5. The long incubation time in connection with high enzyme dosage supported the release of galactose. Therefore the exo-β-1,4-galactosidase activity of the enzyme was investigated. The endo-1,4-β- galactanase from *E. nidulans* was not, however, able to hydrolyse p-nitrophenyl-β-D-galactopyranoside, the compound commonly used to determine the exo-galactosidase activity. Another explanation of generating galactose monomer by endo-1,4-β- galactanase was that this enzyme acted according to the multiple attack mechanism. The theory of the multiple attack mechanism assumes sliding of the enzyme along the substrate polysaccharide chain without dissociation between successive cleavages (Mazur and Nakatani, 1993). The multiple attack mechanism may result in the production of saccharides of different DP, including monomers. Moreover, the high content of galactose was reported also in the products

obtained by hydrolysis with other endo-1,4-β- galactanases, for example in the publication by Yamaguchi et al., 1995.

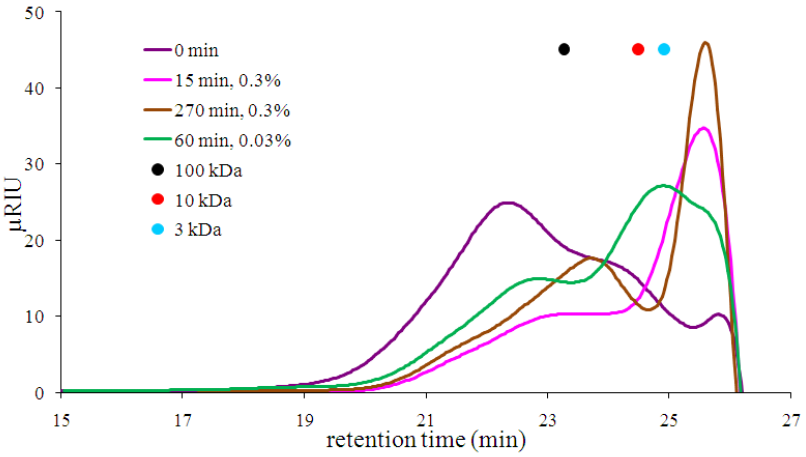

Figure 15. HPSEC analysis of E. nidulans endo-1,4-β-galactanase – catalyzed hydrolysis of potato galactan.

6.2.3 Investigation of prebiotic potential of produced galactosaccharides

The potential prebiotic properties of the produced fractions of poly- and oligosaccharides generated by enzymatic catalysis of SPPP and galactan were evaluated by single cultures growth using a Bioscreen microtiter system. The growth performance expressed as an area under the growth curve (Mäkeläinen et al., 2010) was assessed for three probiotic strains, *L. acidophilus* (NCFM, ATCC 700396), *B. longum* (Bl-05, Danisco Global Culture Collection, DGCC 9917), and *B. lactis* (HN019, DGCC 2013), one pathogenic strain of *C. perfringens* (ATCC 13124), and one commensal strain of *E. coli* (ATCC 11775). Fructooligosaccharides and galactan were used as established prebiotic standards (Ziemer and Gibson, 1998).

The growth of each bacterium was affected differently by the different compounds and different bacteria responded differently to one substrate (Figure 16). The PG5 fraction (<3 kDa, 270 min, E/S 0.3%) stimulated the growth of *L. acidophilus* and *B. longum* significantly better than other products of enzymatic hydrolysis. The stimulation of the growth of *L. acidophilus* was at the same level as that achieved with FOS but significantly better ($p < 0.05$) than that obtained with pure galactose and substrate for enzymatic hydrolysis - galactan. The stimulation of *B. longum* was more than 6-fold better than that by FOS and significantly better ($p < 0.05$) than that achieved by galactose and galactan. On the other hand, the growth of *E. coli* and *C. perfringens* on PG5 was higher than their growth on FOS, although for *E. coli* the same as on galactose and for *C. perfringens* lower than on galactose.

The PG1 fraction (<3 kDa, 60 min, E/S 0.03%) and the SPPP1 (<10 kDa fraction generated by digestion of SPPP for 15 min, E/S 0.3%) stimulated the growth of *L. acidophilus* and *B. longum* to a significant extent too. The stimulation of *B. longum*

growth by PG1 and SPPP1, as well as by PG4 was significantly better than that obtained with either FOS, galactose or galactan ($p < 0.05$). Another positive finding was that the stimulation of growth of *C. perfringens* by samples PG1, PG4 and SPPP1 was lower than the stimulation by FOS. The only drawback of these substrates was relatively high growth of *E. coli* - higher than on FOS.

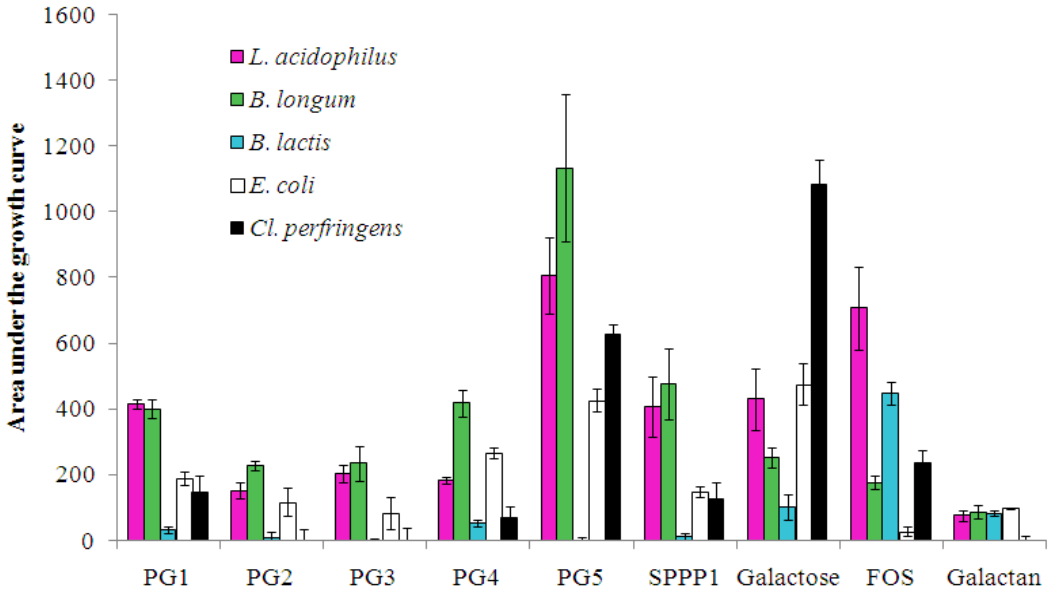

Figure 16. Growth of bacterial strains on *E. nidulans* endo-1,4-β-galactanase catalysed hydrolysis products from potato galactan and SPPP. Substrates were used at concentration of 10 g/L for all bacterial strains. Data are given as average values of 3-6 growth assay replicates and shown ± s.d. Galactose and FOS were used as controls.

The fractions PG1, PG3, PG5, and SPPP1 all contained low molecular weight oligomers and some galactose, which might explain some of the growth stimulation of *L. acidophilus* and *B. longum* promoted by these substrates. However, the growth of *B. longum* was better on all these fractions than on galactose. This may indicate that each of these hydrolysates contained structures, of relatively low molecular weight, which stimulated the growth of *B. longum* very effectively. The data also showed that the growth of both *E. coli* and *C. perfringens* was not supported by low molecular weight samples, besides PG5, as well as by PG2 and PG4 – high molecular weight fractions and galactan.

The high molecular weight fraction SPPP2 was tested at lower concentration than the other samples due to its high viscosity in the solution at this dosage level. Therefore, it is not possible to compare it with the others. It can be, however, concluded that SPPP2 did not support the growth of any of the investigated microorganisms to a high extent.

Analysis of the Bioscreen data showed that among the tested samples the lower molecular weight fractions exhibited more potentially prebiotic features. Whether the observed low or no growth supporting effects on *C. perfringens* have any effect in the

competition among the bacteria in the mixed microbiota in the gut needs further research. The studies in mixed bacterial cultures will enable not only to investigate the stimulation or its lack for pathogens, but also may result in revealing enhanced prebiotic properties of the tested compounds. This depends on the pool of enzymes possessed by the probiotic and pathogenic microorganisms. It seems that the growth of bifidobacteria should be promoted effectively by the galactooligo- and polysaccharides, since these bacteria are often mentioned to possess endo- and exo- galactanase activities. On the other hand, the pathogens may exhibit the same enzyme activities or may benefit from the products of metabolism of bifidobacteria and thus grow well in the gastrointestinal tract. However, the widely described products of bifidobacteria metabolism are SCFA, which should decrease pH in the large intestine and therefore prevent growth of pathogens.

6.3 CONCLUSION

A very active endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was successfully produced in a high yield. It was purified efficiently by affinity chromatography and gel filtration, and the purification enabled crystallization of the enzyme. The crystallization revealed that there were fifteen zinc ions bound to the protein. The enzyme was characterized by determination of its kinetic parameters and thermostability.

The endo-1,4-β-galactanase was used to produce a range of poly- and oligosaccharides from high molecular weight galactan-rich potato fibers (SPPP), as well as from isolated potato galactan. The hydrolysis of polysaccharides by endo-1,4-β-galactanase resulted in many fractions of different molecular size, depending on the applied enzyme amount and reaction time. The products of polysaccharides degradation were separated by filtration. Majority of obtained fractions revealed significant prebiotic potential on single strain cultures.

The production of galactosaccharides from potato pulp, a side-stream from starch manufacturing, demonstrated possibility to generate a new value-added product for an agricultural industry. The release of galactosaccharides from potato waste can be therefore an important step in developing competitive process for new functional food ingredients.

7 MODIFICATION OF SPPP

This chapter is based on un-published results obtained after publication of the journal article described in chapter 6 and attached in chapter 10.3.

7.1 HYPOTHESES AND OBJECTIVES

Hypotheses:

- Pretreatment of SPPP with sodium hydroxide and/or pectin-degrading enzymes makes it more susceptible to the action of endo-1,4-ß-galactanase from *E. nidulans*.
- Multistep modification of SPPP can be used to generate prebiotics.

Objectives:

- Multi-step treatment of SPPP with sodium hydroxide, pectin-degrading enzymes, and endo-1,4-ß-galactanase to release oligosaccharides, followed by compositional and MW analysis.
- To investigate prebiotic potential of the products.

7.2 RESULTS AND DISCUSSION

7.2.1 Chemical and enzymatic modification of SPPP

The incubation of SPPP with endo-β-1,4-galactanase from *E. nidulans* resulted in two product fractions (Figure 14), as mentioned in section 6.2.2. The first one of molecular weight below 10 kDa contained 93% of galactose. The second one of MW higher than 10 kDa was composed of only 54% of galactose. The other components of the high MW fraction were arabinose, galacturonic acid, glucose, mannose and rhamnose, which constituted 20, 11, 7, 5, 3%, respectively. As can be seen from the composition, the fraction of MW above 10 kDa was mainly derived from arabinogalactan. However, the high content of galacturonic acid indicated that the rhamnogalacturonan backbone was present as well and because of the high MW of this fraction, it could be assumed that RGI was not degraded. It was hypothesized that the degradation of the residual RGI backbone in SPPP would make the substrate better available for endo-galactanase enabling more complete hydrolysis of galactan by this enzyme. This would result in higher release of lower MW products, which exhibited higher prebiotic potential. It would also support better utilization of the material and thus generation of better value-added product from potato pulp. The degradation of the backbone of RGI before applying endo-β-1,4-galactanase could also result in the products of different composition than the ones reported before. The new composition of

galactooligosaccharides could influence the prebiotic properties of these compounds. Following this idea, other enzymes could be applied to modify oligosaccharides from SPPP. Arabinofuranosidase and exo-polygalacturonase could potentially remove arabinose and galacturonic acid from the substrate and, as an outcome, galactose content in the product would rise.

The scheme of all the modifications to which solubilized potato pulp polysaccharides were subjected is shown in Figure 17 and the names of produced samples are denoted in Table 9. At first the substrate was treated with α- amylase from *B. licheniformis* (TermamylSC) in order to remove the residual starch present in the material. Incubation with this enzyme resulted in release of glucose and glucooligosaccharides, which were removed from SPPP by filtration with 2 kDa membrane.

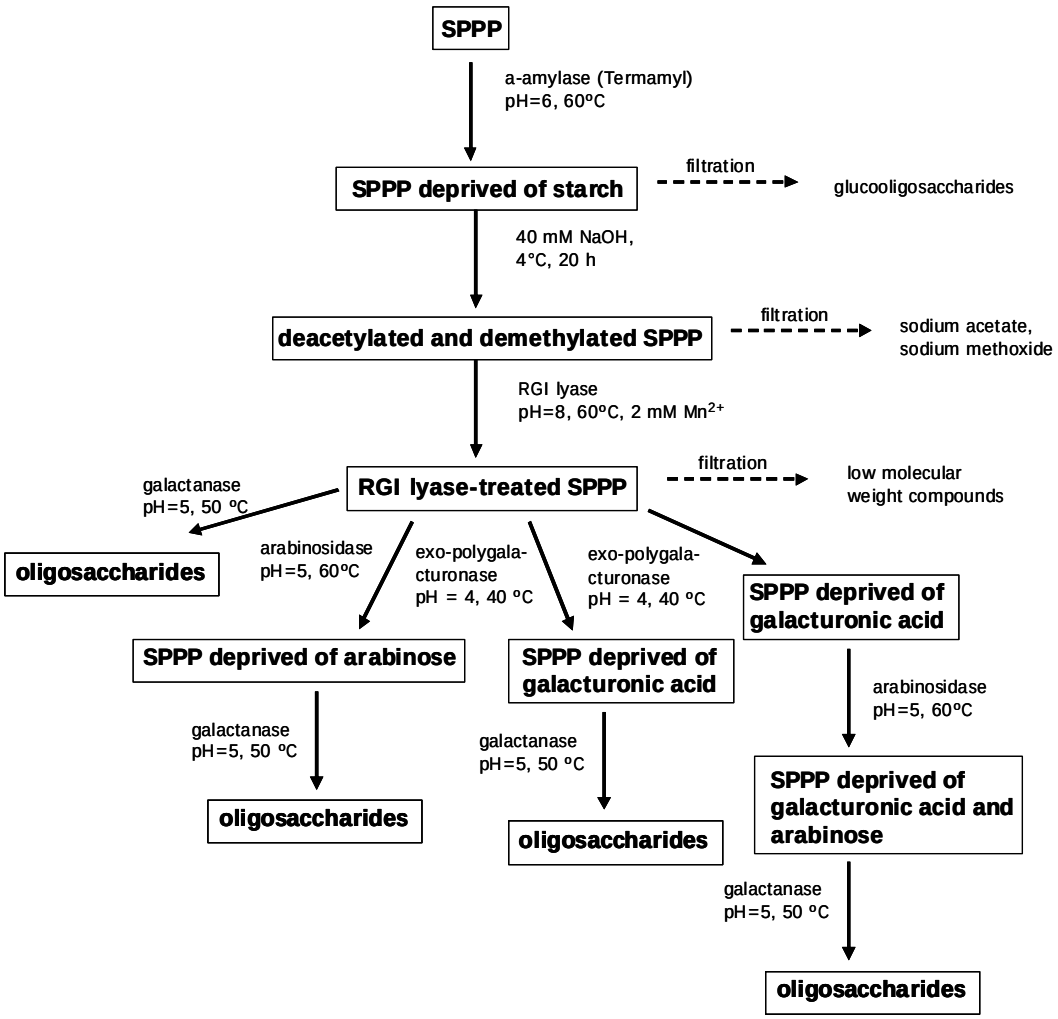

Figure 17. Scheme of modifications of SPPP.

Sample	Name
Crude SPPP	0
SPPP treated with α-amylase	1
SPPP treated with α-amylase and NaOH	2
SPPP treated with α-amylase, NaOH and RGI lyase	3
SPPP treated with α-amylase, NaOH, RGI lyase and endo-galactanase	4
SPPP treated with α-amylase, NaOH, RGI lyase, α-arabinosidase and endo-galactanase	5
SPPP treated with α-amylase, NaOH, RGI lyase, exo-polygalacturonase and endo-galactanase	6
SPPP treated with α-amylase, NaOH, RGI lyase, exo-polygalacturonase, α-arabinosidase and endo-galactanase	7

Table 9. The name codes of produced SPPP derivatives.

Some preliminary experiments performed to loosen the structure of RGI present in SPPP revealed that treatment of SPPP with RGI lyase from *Bacillus licheniformis* resulted only in a slight profile change in its size exclusion chromatogram (Figure 18). That was not considered as satisfying degradation of RGI backbone of SPPP. Therefore sodium hydroxide was applied to remove acetyl and methyl groups present in RGI structure (Sun et al., 1987; Normand et al., 2010), since the acetylation and methylation may hinder the action of RGI lyase. After deesterification of galacturonic acid contained in RGI by incubation with NaOH, size exclusion chromatogram of SPPP did not indicate any change in the molecular weight (Figure 18). However, combining incubation of SPPP with NaOH and subsequently with RGI lyase resulted in significant decrease of molecular size of SPPP (Figure 18) indicating that the ester moieties obstructed the degradation of RGI by RGI lyase from *B. licheniformis*. This is consistent with literature. For example, activity of RGI lyase from *Aspergillus aculeatus* decreases when the degree of acetylation of the substrate increases (Mutter et al., 1998).

In the next steps of SPPP degradation three carbohydrate-degrading enzymes were used in different combinations. SPPP was divided into four samples. The first one was treated with endo-1,4-β-galactanase and poly- and oligosaccharides of molecular weight below 10 kDa were produced. The second sample was incubated at first with α- L-arabinofuranosidase resulting in release of arabinose detected by HPAEC and subsequently with endo-1,4-β-galactanase resulting in formation of poly- and oligosaccharides. The third sample was treated at first with exo-polygalacturonase creating free galacturonic acid and subsequently with endo-1,4-β-galactanase forming

again poly-and oligosaccharides. The fourth was subjected to action of exo-polygalacturonase, then α-L-arabinofuranosidase and eventually endo-1,4-β-galactanase. That resulted in release of monosaccharides and subsequently galactosaccharides of different chain length. The monosaccharides produced by the incubation with arabinofuranosidase and exo-polygalacturonase were removed by filtration prior to employing endo-galactanase. The incubation with exo-polygalacturonase was done to remove terminal galacturonic acid residues, still present in the RGI component of SPPP.

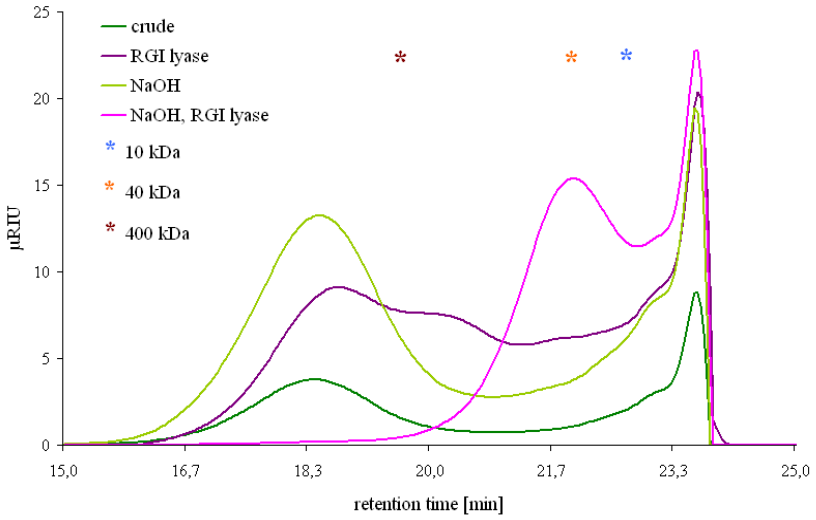

Figure 18. HPSEC analysis of SPPP modification by sodium hydroxide and RGI lyase from *Bacillus licheniformis*.

In general, the degradation of the NaOH and RGI lyase- treated SPPP with endo-1,4-β-galactanase from *E. nidulans* resulted in a range of products of molecular weight mainly between 1 and 10 kDa (samples 4 -7). Whereas treatment of crude SPPP with this enzyme gave two ranges of products: one of them of MW between 1 and 10 kDa and the second above 100 kDa. The fraction above 100 kDa contained high level of galacturonic acid. This indicated that the RGI remained intact when NaOH and RGI lyase were not used.

The treatment of SPPP, which was earlier modified by deesterification and RGI lyase, with galactanase, as well as with arabinosidase and/or exo-polygalacturonase and subsequently with galactanase gave the same size-exclusion profile of poly- and oligosaccharides (Figure 19). The monosaccharide composition of crude and modified SPPP– after each step of modification is summarized in Table 10. The composition was investigated by acid hydrolysis of the materials and analysis by HPAEC. The main ingredient of all the samples was galactose. Its content increased after application of amylase, because α-amylase removed the residual starch and therefore decreased the content of glucose in the material. However, glucose content was still relatively high in the sample incubated with α-amylase. That can be attributed to a drawback of filtration, which did not provide full separation. In the sample coming from the next step of the

process the glucose content was much lower – due to the second filtration of low MW compounds, and it stayed at the same level until the end of SPPP processing. The content of mannose increased in the process of SPPP modification. This was connected to the presence of mannose in the structures of applied enzymes. The enzymes were not removed from the post-reaction mixture when acid hydrolysis was performed to assess the monosaccharide composition. With an addition of each enzyme, the content of mannose increased. It happens often that enzymes expressed in *P. pastoris* are glycosylated with mannose (Macauley-Patrick et al., 2005).

A surprising result was a presence of fucose in the hydrolysed material. It was not detected in the study where SPPP was treated with endo-1,4-β-galactanse alone (publication attached in chapter 9.3). However, another batch of potato pulp was used in that study. The literature also mentions that fucose may be present in potato RGI (Byg et al., 2012).

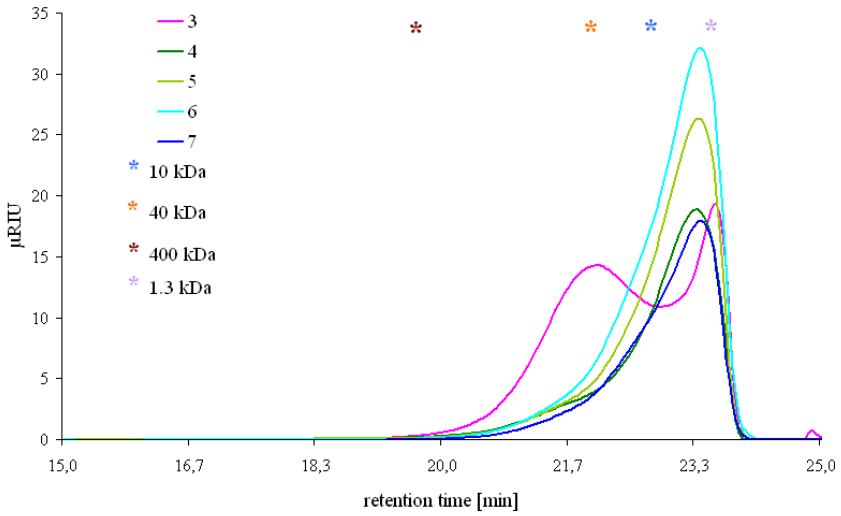

Figure 19. HPSEC analysis of modification of SPPP with α-amylase from *B. licheniformis*, sodium hydroxide, RGI lyase from *B. licheniformis*, endo-1,4-β-galactanse from *E. nidulans*, α-L- arabinofuranosidase from *E. nidulans*, exo-polygalacturonase from *E. nidulans*.

Sample	Composition [mol %]						
	Fucose	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Galacturonic acid
0	2.0±0.4	3.7±1.8	14.±0.4	64.0±6.1	11.3±0.4	3.2±0.4	8.6±3.9
1	1.8±0.1	3.1±0.6	15.2±0.3	67.4±1.6	7.3±0.3	3.0±0.1	7.2±1.1
2	1.8±0.3	4.6±0.9	14.3±0.7	62.8±1.8	6.3±0.1	3.5±0.2	10.6±1.6
3	1.6±0.2	4.9±0.1	12.2±0.7	66.9±0.9	5.5±0.2	8.8±0.2	5.4±2.1
4	1.9±0.6	3.8±0.1	11.1±0.4	69.3±2.0	3.9 ±0.1	10.0±0.6	4.6±1.5
5	2.1±0.2	3.8±0.3	10.2±0.4	68.2±3.0	3.5±0.1	10.8±0.7	5.8±2.3
6	2.0±0.0	3.7±0.2	10.3±0.2	66.9±0.5	3.5±0.1	12.0±0.3	6.0±0.3
7	2.1±0.4	3.8±0.3	10.4±0.6	67.4±1.8	3.4±0.1	12.8±0.3	4.4±1.1

Table 10. Composition of products of SPPP modifications.

7.2.2 Investigation of prebiotic properties of modified SPPP

The growth of probiotic bacteria including species of *B. longum*, *B. longum* subsp. *infantis*, *B. lactis* and *L. acidophilus*, as well as a pathogen, *C. perfringens* was investigated by measurement of optical density at 600 nm. The growth of each bacterium was assessed on crude and modified SPPP (Figure 20). *B. infantis* 233 grew to the highest extent among all the bacteria on the samples treated with endo-galactanase (samples 4, 5, 6, 7), but it did not grow well on pure galactan used as a reference prebiotic. *L. acidophilus*, on the other hand, grew well on all endo-galactanase- modified samples and on galactan.

A few of probiotics used sample 2 as a carbon source. Those were *B. longum* 232, *B. infantis* 233, *B. infantis* 1497 and *B. lactis*. Particularly *B. infantis* 1497 grew relatively well on this sample. It grew also to some extent on crude SPPP and samples 1 and 3. On the contrary to the other strains, *B. infantis* 1497 grew on samples 0-3 to, more or less, the same extent as on samples 4-7. It could be therefore speculated that there was a structure motif in all these samples that was vulnerable to enzymes produced by the *B. infantis* 1497 strain.

All the prebiotic strains were stimulated by the SPPP fractions modified with endo-galactanase and thus showed a prebiotic potential. In general, there was not a significant growth stimulation difference between the specific samples incubated with galactanase, i.e. samples 4, 5, 6 and 7. The modifications of SPPP caused by the action of α-

arabinosidase and exo-polygalacturonase did not influence the bacterial growth to high extent.

C. perfringens was stimulated by the produced compounds, whereas it was not stimulated by galactan. The fact that *C. perfringens* did not grow on galactan, but grew to some extent on SPPP treated with endo-galactanase is in agreement with the previous findings (section 6.2.3). The sample of MW below 10 kDa obtained by treatment of crude SPPP with endo-1,4-β-galactanase from *E. nidulans* stimulated the growth of *C. perfringens* to an extend when its area under the growth curve reached approximately 130 units (Figure 16, section 6.2.3.). In the present experiments the areas under the growth curve for *C. perfringens* were between 41 for sample 5 and 151 for sample 6. It can be, therefore, concluded, as in section 6.2.3., that mixed cultures study is required to investigate cross-feeding effects between strains.

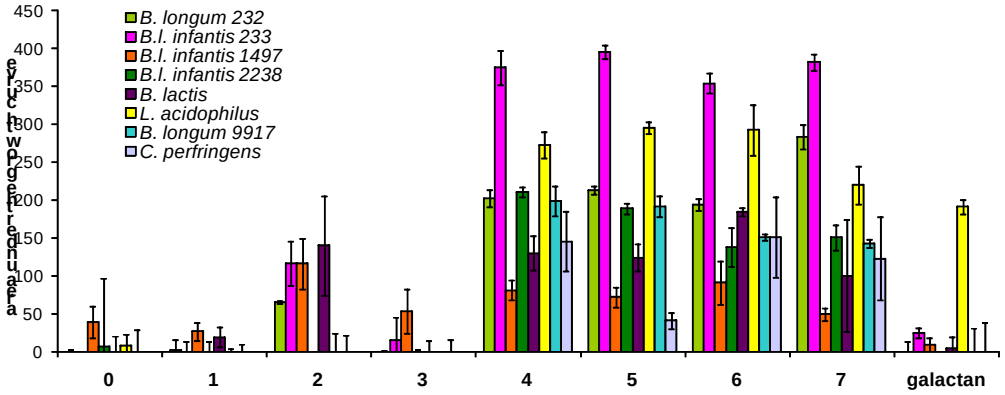

Figure 20. Differential growth of bacterial strains on fractions of modified SPPP; galactan was used as a control; growth responses for all the substrates are shown for a substrate concentration of 10 g/L for all bacterial strains. Data are given as average values of 3 growth assay replicates and shown ± S.D.

7.3 CONCLUSION

The treatment of SPPP with sodium hydroxide and subsequent incubation with RGI lyase from *B. licheniformis* resulted in decrease of molecular size of SPPP from above 400 kDa to 40 kDa, whereas the treatment of SPPP exclusively with RGI lyase changed the molecular size of the substrate only slightly, since it was still bigger than 400 kDa. The chemical deesterification of rhamnogalacturonan I contained in SPPP improved the efficiency of RGI lyase and enabled successful degradation of RGI present in the material. The application of RGI lyase in the process of production of galactopoly- and oligosaccharides of narrower molecular weight was successful, because galactosaccharides generated by endo-1,4-β-galactanase from *E. nidulans* from NaOH and RGI- treated material were within a relatively narrow range of 1 - 10 kDa, whereas the incubation of the crude SPPP with endo-1,4-β-galactanase (described in section 6.2.2) resulted in a much broader range of products, i.e. in two distinct fractions: one of

MW around 3 kDa and the second one about 400 kDa. Moreover, the products of SPPP modifications and subsequent incubation with endo-1,4-β-galactanase revealed prebiotic potential on single strain cultures.

8 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Two types of potentially prebiotic oligosaccharides were produced in enzyme-catalysed reactions. The first type of produced oligosaccharides was sialylated oligosaccharides obtained in reaction catalysed by mutated sialidases. The sialylated oligosaccharides represented structures of human milk oligosaccharides, as well as other glycans. The HMOs were synthesized by transfer of sialic acid from casein glycomacropeptide, a dairy side stream, catalysed by mutated sialidase of *T. rangeli* possessing six point mutations, Tr6. The other sialylsaccharides, human milk- like oligosaccharides were generated by mutant enzyme with thirteen point mutations, Tr13. The substrates for this reaction were compounds of established prebiotic properties, compounds similar in structure to lactose – a model substrate in synthesis of HMOs, and monomers such as fucose and glucose. Obtained products were purified by anion exchange chromatography. Prebiotic potential of human milk-like oligosaccharides was tested in the single cultures experiments.

The second type was galactooligosaccharides which were produced from commercially available galactan and from an agricultural waste, i.e. potato pulp. The potato pulp was modified by different methods, mainly enzymatic, prior to release of galactooligosaccharides by the action of an endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris*. Together with oligosaccharides compounds of higher MW were released. The products were fractionated by membrane systems and their prebiotic potential was investigated by incubation with single bacterial strains. The galactosaccharides revealed promising prebiotic potential.

The results of this work pave the way to develop new functional food ingredients. The mutant sialidase from *T. rangeli*, Tr6 has been successfully tuned into trans-sialidase and a few structures of human milk oligosaccharides were produced by this enzyme. Application of another *T. rangeli* enzyme with 13 point mutations, Tr13 for the synthesis of sialylated glycans provides an opportunity to produce a range of novel oligosaccharides. These new sialylated glycans could be used in infant formula as potent prebiotics, and to combat the food-borne pathogens by adhesion (decoy molecules). Use of Tr13 ensures not only relatively high reaction yields but also, on the contrary to production of HMO, possibility of employing cheap acceptor compounds. Moreover, this work has also provided a positive foundation for biocatalytic design of β-galactooligosaccharides from potato pulp with potential health effects. It has also shown a direction for potential valorisation of the potato pulp by-product.

The present work resulted in generation of potential value-added products from side-streams from agriculture and dairy industry. Sialylated oligosaccharides and galactosaccharides are compounds of great biological and market potential. They are recognized as prebiotics, immune system stimulators and factors preventing infections.

GOS are already available on the market but the alternative way of their production and possibility to produce compounds of DP higher than nine may be a valuable asset. A possibility of adding HMOs to the infant formulas makes these products novel and significantly improves their properties and market image. The production of HMOs may also add up an opportunity to create new products, which would have not only a nutritional and prophylactic, but also therapeutic properties. The HMO-like saccharides could be used in products for both infants and adults. Their production should be in some cases cheaper than production of the real HMOs.

The oligosaccharides described above have to be studied further before they can be used commercially. Adjustment of their production process to the requirements of the industrial scale needs also further effort. The prebiotic properties should be confirmed in mixed cultures fermentations and human trials. Especially, the biological properties of potential ingredients of infant formulas require more investigation. Furthermore, their purity should be ensured at very high level. Therefore the chromatographic method of purification should be exchanged to another one or altered, e.g. by use of another buffer than ammonium formate, which is not easy to remove from the product, or by enhancement of the buffer removal step. The characterization and purification of the donor substrate, cGMP should be improved as well. The development of HMO production process could be accomplished by reuse of enzyme and employing a membrane reactor. Since the trials of immobilization of the mutant sialidase Tr6 done at our department recently did not result in an improved performance, the membrane reactor seems to be a promising solution. Especially continuous system could promote the process by steady removal of the product by filtration. The fouling caused by the cGMP would have to be diminished in that case and the low molecular weight acceptor used in the process would have to be supplied continuously, since it would leave the reactor together with the low molecular weight product of the reaction. One more problem connected to HMOs production which has to be addressed is the price of the sialic acid acceptors which, besides lactose, are very expensive. Their cheaper source should be sought. An additional method of improving the economics of production of sialylated glycans could be use of residual, sialic acid- deprived cGMP to generate another useful product, for example, biofuels.

The mutated sialidases could be manipulated further at the gene level to achieve enzyme possessing exclusively trans-sialidase activity. That would enable use of the same molar concentrations of donor and acceptor in the reaction, because the product would not be hydrolysed any more and the excess of the acceptor would not be required any longer. Thermostability is also a desired feature of trans-sialidase. The application of thermostable trans-sialidase would improve the substrates solubility and prevent the contamination of the reaction mixture. Moreover, the promiscuity of Tr13 could be tested further and other interesting sialylated saccharides could be obtained.

The scale of galactosaccharides production should be also gradually enlarged in case of industrial interest in the production of these compounds from the potato pulp. A continuous membrane reactor appears to be a good solution for that purpose, since the

steady state created in this type of equipment would enable release of products of narrow MW and would prevent degradation of GOS to monosaccharide catalysed by the endo-1,4-β-galactanase. The immobilization of the enzyme could be tested as well.

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10 PUBLICATIONS

10.1 Biocatalytic production of 3'-sialyllactose by use of a modified sialidase with superior trans-sialidase activity

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Biocatalytic production of 3′-sialyllactose by use of a modified sialidase with superior *trans*-sialidase activity

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ABSTRACT

Casein glycomacropeptide (cGMP) and lactose, which are purified (or semi-purified) components obtained from side streams from dairy industry operations, were used as substrates for enzyme catalyzed production of 3′-sialyllactose, a model case compound for human milk oligosaccharides (HMOs). The enzyme employed was a mutated sialidase, Tr6, derived from *Trypanosoma rangeli*, and expressed in *Pichia pastoris* after codon-optimization. The Tr6 contained 6 point mutations and exhibited *trans*-sialidase activity. The Tr6 *trans*-sialidase reaction conditions were tuned for maximizing Tr6 catalyzed 3′-sialyllactose production by optimizing pH, temperature, acceptor, and donor concentrations using response surface designs. At the optimum reaction conditions, the Tr6 catalyzed the transfer of sialic acid from cGMP to lactose at high efficiency without substantial hydrolysis of the 3′-sialyllactose product. The robustness of the Tr6 catalyzed reaction was verified at 5 L-scale providing a yield of 3.6 g 3′-sialyllactose at an estimated molar *trans*-sialylation yield of 50% on the 3′-sialyl in cGMP. Lacto-*N*-tetraose and lacto-*N*-fucopentaoses also functioned as acceptor molecules demonstrating the versatility of the Tr6 *trans*-sialidase for catalyzing sialyl-transfer for generating different HMOs. The data signify the applicability of enzymatic *trans*-sialylation on dairy side-stream components for production of human milk oligosaccharides.

1. Introduction

Human milk oligosaccharides (HMOs) appear to constitute an innate immunologic mechanism by which human milk confers breast-fed infants some level of protection against infections [1]. HMOs constitute the third most abundant component of human milk after lactose and lipids, and levels of up to 8 g/L have been reported [2], mainly during the first days of lactation. The HMOs function as soluble receptors that inhibit pathogens such as *Campylobacter jejuni* and *Escherichia coli* K1 from adhering to their target receptors on the mucosal surface of the host gastrointestinal tract, and thereby help reduce the incidence of diarrhea and other diseases in breast-fed infants [1,3]. HMO molecules are also substrates for probiotic gut bacteria, including *Bifidobacterium longum subsp. infantis* [4] (previously known as *Bifidobacterium infantis*) and thus seem to support the development of a healthy gut microflora in breast-fed infants by acting as bifidogenic prebiotics [4].

All HMO molecules contain lactose (Galβ1-4Glc) at their reducing end and are built from sequential elongations via linkage to one or more units of *N*-acetylglucosamine and galactose (*N*-acetyllactosamine) and can be decorated with several sialic acid (*N*-acetyl-neuraminic acid) and fucose residues [1,2,5]. Because of the different backbone lengths, bonds, decorations via various bond types, and different combinations of the basic building blocks many different HMO structures can exist. About 180 different HMO species have thus been identified in a pooled human milk sample from five individuals, and nearly 16% of the total oligosaccharide abundances were found to correspond to sialylated oligosaccharides [5]. Only a few HMOs are present in bovine milk and only at low concentrations.

Abbreviations: cGMP, casein glycomacropeptide; HMOs, human milk oligosaccharides; LNT, lacto-*N*-tetraose; LNnT, lacto-*N*-neotetraose; LNFP I, lacto-*N*-fucopentaose; LNFP V, lacto-*N*-fucopentaose V.

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Bifidobacterium longum subsp. infantis (ATCC15697) is considered an "archetypical HMO-utilizing bacterium" [6], and has been found to harbor genes for sialidase and fucosidase glycosyl hydrolases [6]. Exo-α-sialidase activity (EC 3.2.1.18) has also been positively demonstrated in two strains of *Bifidobacterium bifidum* (JCM1254 and JCM7004) [7].

Enzymatic synthesis (analytical scale) of sialylated oligosaccharides by use of the *trans*-sialidase from the pathogenic parasite *Trypanosoma cruzi* was reported already in 2000 [8]. The *T. cruzi trans*-sialidase has been studied intensively due to its suggested involvement in the mammalian host cell invasion process and pathogenesis of *T. cruzi* leading to Chagas disease [9]. Hence, recombinant, catalytic domain forms of this enzyme have been expressed in *E. coli* [10,11] and it has also been reported that the recombinant enzyme can catalyze the sialylation of lactose derivatives such as lactitol and lactobionic acid using 3′-sialyllactose (α-Neu5Ac-(2-3)-gal-β-(1-4)-glc) as donor [12]. The recombinant version of the *T. cruzi* enzyme expressed in *E. coli* has also been used for preparative sialylation of a synthetic pentasaccharide from the mucins of *T. cruzi* [13] and methods for enriching bovine milk with 3′-sialyllactose employing the *T. cruzi trans*-sialidase and claims for using a sialidase from other organisms have also been patented [14,15]. The full *T. cruzi* dual-domain *trans*-sialidase enzyme is considered a virulence factor for the invasive *T. cruzi* phenotype [16], but the recombinant *T. cruzi trans*-sialidase used in synthesis would not be virulent, and would moreover be inactivated when heating and separation steps are included in the *trans*-sialylation process. Nevertheless, due to its origin from a pathogenic organism it may be difficult to obtain approval and consumer acceptance for using the *T. cruzi trans*-sialidase as an aid for producing functional food ingredients for infant consumption. Regarding catalysis facilitated by sialidases, i.e. sialidase glucosyl hydrolases (neuraminidases) (EC 3.2.1.18), the problem is that the reaction will invariably result in hydrolysis of the target 3′-sialyllactose product.

However, mutants of the sialidase from the non-pathogenic *Trypanosoma rangeli* have been reported to exhibit low *trans*-sialidase activity (maximum 11% of the *trans*-sialidase activity of the *T. cruzi trans*-sialidase) [17]. We hypothesized that the use of such a sialidase mutant from *T. rangeli* exhibiting *trans*-sialidase activity could provide an alternative route for enzymatic, biomimetic in vitro synthesis of HMOs. The present study was undertaken to assess the catalytic ability of such a *T. rangeli* derived *trans*-sialidase mutant for (i) enzymatic in vitro production of gram-levels of 3′-sialyllactose, and (ii) enzymatic sialyl-transfer to other HMO structures such as lacto-*N*-tetraose and lacto-*N*-fucopentaoses. An additional objective was to utilize abundantly available food-grade co-processing products from the dairy industry, i.e. lactose and casein glycomacropeptide (cGMP), as substrates for the enzymatic reaction to make the process relevant in relation to the prospective use of sialylated HMOs as functional food ingredients.

2. Materials and methods

2.1. Substrates

β-D-lactose and standard of *N*-acetylneuraminic acid (sialic acid) were purchased from Sigma–Aldrich (Steinheim, Germany). Standards of 3′-sialyllactose and 6′-sialyllactose were purchased from Carbosynth (Compton, United Kingdom). Lacto-*N*-tetraose (LNT), Lacto-*N*-neotetraose (LNnT), Lacto-*N*-fucopentaose I (LNFP I), and Lacto-*N*-fucopentaose V (LNFPV) were purchased from Elicityl SA (Crolles, France). Casein glycomacropeptide (cGMP) in the form of the commercially available product Lacprodan® CGMP-20 (intended for use in infant formulas and PKU-products), containing a total of 5.7% (w/w), equivalent to 0.2 mmol/g dry matter, of covalently linked sialic acid was a gift from Arla Foods Ingredients amba (Viby, Denmark). Before use, as a technical precaution in relation to HPAEC-analysis, low molecular weight impurities in the cGMP solution were removed by filtration on a 5 kDa membrane (Sartorius AG, Goettingen, Germany).

2.2. Vector and strain construction

A *Trypanosoma rangeli* sialidase gene (Genbank accession no. U83180.1) with the following mutations, M96V, A98P, S120Y, G249Y, Q284P and I37L [17] was codon-optimized and synthesized by DNA 2.0 (Menlo Park, CA, USA). The gene was inserted into pPICZαC (Invitrogen, Life Technologies Corp. Carlsbad, CA, USA) between the *Xba*I and *Xho*I restriction sites generating a translational fusion to the α-factor signal sequence and C-terminal c-myc and 6xHis tag. The vector was named pPicZαC_Tr6 (the gene sequence is available in supplementary material Figure S1). Transformation and selection of *Pichia pastoris* X-33 expressing the Tr6 gene were carried out according to the manufacturer's instructions. Zeocin resistant transformants were analyzed for protein expression by Western blot using a *c-myc* antibody [18].

2.3. Production of the Tr6 enzyme in P. pastoris, purification and characterization

The enzyme, Tr6, was produced in a 5 L Sartorius Biostat Aplus fermentation of *P. pastoris*, in a fermentation run principally as detailed previously [19], with the exception that the methanol fed-batch phase was done at 25 °C. The Tr6 enzyme was purified by Cu^{2+} affinity column chromatography using a CIM® IDA-8f mL Tube Monolithic Column (BIA Separations GmbH, Villach, Austria) [18]. The protein concentration was measured at 280 nm using Gene5™TAKE3 module version 1.09 according to the manufacturer's instructions (Biotek® Instruments, Inc.).

The purity of the Tr6 was evaluated by SDS-PAGE (BioRad, CA, US) using Coomassie brilliant blue staining as described previously [18]. To test for *N*-glycosylation the purified Tr6 was treated with endoglycosidase H (EndoH, Medinova) for 1 h at 37 °C. Proteins treated with and without endoglycosidase H were analyzed by Western blot, using monoclonal anti-poly-histidine–peroxidase antibodies, principally as detailed previously [18].

2.4. Investigation of conditions for trans-sialylation by statistically designed experiments

MODDE Version 7.0.0.1 (Umetrics AB, Umeå, Sweden) was used as a tool to design the experimental frame, a quadratic central composite design, and to fit and analyze the data by multiple linear regression analysis. Lactose was used as an acceptor and cGMP as donor of sialic acid. The buffer used in the reactions was 15 mM phosphate-citrate buffer with pH values indicated below. Tr6 was incubated at a concentration of 5.3 mg/L. Three pH regimes 5.5, 6.5 and 7.5 were tested. Incubation temperatures were 15, 20 and 25 °C. Lactose concentration varied from 0.34 to 351 mM and concentration of sialic acid residues bound in cGMP varied from 0.6 to 4.6 mM in the experimental design frame: lactose and cGMP were solubilized in buffer and pre-incubated at specific temperatures, before the reactions were initiated by addition of enzyme. The biocatalysis process was allowed to proceed for 20 min, and then stopped by heating for 10 min at 90 °C. Subsequently each sample was filtered on a 5 kDa polyethersulfone membrane to remove cGMP (Vivaspin, Sartorius AG, Goettingen, Germany). Concentrations of 3′-sialyllactose and sialic acid were determined by HPAEC and/or LC/MS as described below.

2.5. Time study of trans-sialylation catalyzed by Tr6 expressed in P. pastoris

Tr6 was incubated as described above at two different conditions: 25 °C, pH 5.5 with 36.8 mM lactose and 2.6 mM sialic acid bound in cGMP or with 117 mM lactose and 4.6 mM sialic acid bound in cGMP. The reaction was followed for 100 min.

2.6. Enzymatic production of 3′-sialyllactose

The enzymatic *trans*-sialylation reaction was accomplished in a stirred reactor vessel in a total reaction volume of 5 L. 117 mM lactose and 4.6 mM sialic acid linked to cGMP was dissolved in 15 mM phosphate–citrate buffer (pH 5.5), and after pre-incubation in the buffer at 25 °C for 10 min, 5.3 mg/L Tr6 was added. The enzymatic catalysis lasted 20 min, then the reaction was terminated by inactivation of the enzyme at 90 °C for 10 min. cGMP was removed from the reaction mixtures by a cross-flow filtration using a 5 kDa cutoff membrane (Sartorius AG, Goettingen, Germany). The permeate containing 3′-sialyllactose, sialic acid, lactose and buffer was collected and freeze-dried. After re-solubilization in water (775 mL) the reaction products were separated by anion exchange chromatography using a HiScale 50/20 column (GE Healthcare) with a packed bed of Sepharose Q FF on an ÄKTA purifier 100 work station. The elution was monitored at 210 nm. Before injection the column was equilibrated with 2 L of water. After injection of the sample, the column was washed with 1.2 L water and the products 3′-sialyllactose and sialic acid were then eluted with 1.4 L of 40 mM ammonium formate. The products were lyophilized and residual ammonium formate was removed by repeated solubilization and lyophilization.

2.7. Production and purification of sialylated lacto-N-tetraoses and lacto-N-fucopentaoses

The reactions were carried out at 5–100 mL scale employing the same reaction conditions as described for 3′-sialyllactose production except that LNT, LNnT, LNFP

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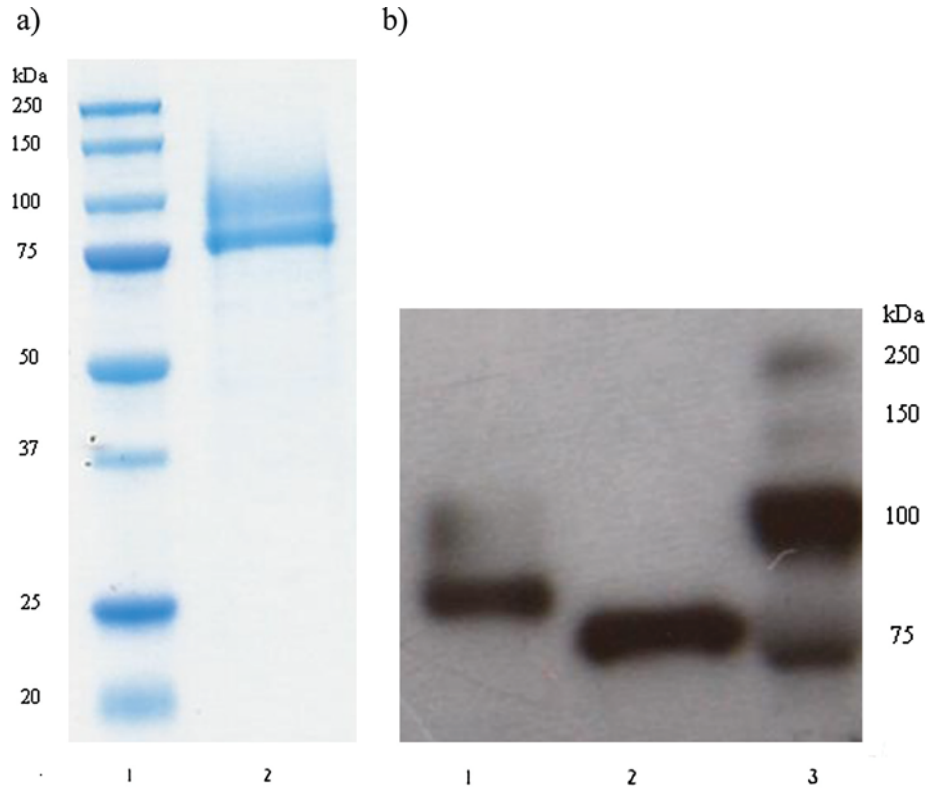

Fig. 1. (a) Coomassie stained SDS-polyacrylamide gel. Lane 1: marker, lane 2: the Tr6 enzyme after IMAC purification. (b) Western blot analysis of the produced Tr6 enzyme: lane 1, crude protein; lane 2, protein after treatment with EndoH; lane 3, marker. For the Western blot proteins were incubated with monoclonal anti-poly-histidine-peroxidase antibodies (Sigma–Aldrich) (as detailed in [18]).

I and LNFP V were used as acceptors instead of lactose. LNT was used at 117 mM, whereas LNnT, LNFP I and LNFP V were used at 25 mM.

2.8. High-performance anion exchange chromatography–pulsed amperometric detection (HPAEC–PAD)

Separation and quantification of 3′-sialyllactose and sialic acid were carried out by HPAEC–PAD analysis using a CarboPac™ PA1 (4 mm × 250 mm) column and a Dionex BioLC system. The eluent system comprised deionised water (A), 0.5 M NaOH (B) and 1 M NaOAc (C) and the elution program was modified from the method described in [20] as follows: For the first 3 min a ratio of 80: 20 (% A:B) was applied, then a linear gradient from 80:20 (% A:B) to 60:20:20 (% A:B:C) was used during 3–27 min. Strongly retained anions were removed from the column by isocratic elution at 40:20:40 (% A:B:C) from 27 to 31 min. Subsequently the column was re-equilibrated for 7 min with 80:20 (% A:B).

2.9. Capillary liquid chromatography/mass spectrometry

For liquid chromatography/mass spectrometry (LC/MS) analyses, an Agilent 1100 LC/Agilent 6340 ion trap MS system was used. Oligosaccharides were separated using a Hypercarb porous graphitic carbon (PGC) column (0.32 × 150 mm, 5 μm, Thermo Scientific) at 30 °C. Samples (0.5 μL) were loaded onto the column in 10 mM ammonium bicarbonate. Gradient elution was achieved using a binary solvent system consisting of (A) 10 mM ammonium bicarbonate, adjusted to pH 8.5 with ammonium hydroxide, and (B) 100% acetonitrile at a flow rate of 5 μL/min. The gradient was initially at 98:2 (% A:B) for 5 min, followed by a linear increase to 42:58 (% A:B) at 33 min. This concentration of B was held for 3 min. Subsequently the eluent was returned to 98:2 (% A:B) at 40 min and the system was allowed to equilibrate for 10 min prior to the next injection. All solvents used were of the highest HPLC grade. The mass spectrometry was performed in negative ion mode, and was scanned in the range *m/z* 150–2200 (2 microscans, maximum accumulation time of 150 ms, an ion current count of 200,000) followed by data-dependent MS2 scans of the four most abundant ions in each MS1 scan.

2.10. Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded in D_2O (99.9% D, Sigma–Aldrich) at 25 °C on a Varian Unity Inova 500 MHz spectrometer equipped with a 5 mm probe, using standard 1D and 2D pulse sequences. 1,4-dioxane was used as an external chemical shift reference (δ_H 3.75 ppm and δ_C 67.4 ppm).

3. Results

3.1. Expression in P. pastoris and characterization of the trans-sialidase Tr6

Since it is generally accepted that codon usage can affect (i.e. decrease) recombinant protein yields [21] a synthetic gene with a codon distribution optimized for *P. pastoris* expression [22] was synthesized for the production of the *T. rangeli* sialidase Tr6. The secreted Tr6 polypeptide comprised 663 amino acids including a 23 amino acid C-terminal c-myc and 6xHis tag (Supplementary material, Figure S1) with a theoretical molecular weight of 73 kDa. A positive transformant expressing the Tr6 enzyme was selected and used for enzyme production at a 5 L-scale. This production gave a concentration of Tr6 of 1 g/L. After purification, the enzyme had an apparent molecular weight of about 80 kDa by SDS-PAGE (Fig. 1a), but treatment of the Tr6 enzyme with EndoH followed by Western blot analysis revealed that the expressed protein was glycosylated and the molecular weight of Tr6 was 75 kDa after deglycosylation (Fig. 1 b).

3.2. Tuning of the Tr6 reaction conditions for maximizing trans-sialidase activity

The wild type sialidase from *T. rangeli* and the derived Tr6 mutant enzyme have an inherent high level of sialidase activity, but a relatively low level of *trans*-sialidase activity [17]. In the present study we wanted to evaluate the relationship between Tr6 sialidase and *trans*-sialidase activities and define the reaction conditions at which the *trans*-sialidase activity would be maximal. For production of 3′-sialyllactose, a systematic evaluation of the influence of the reaction factors, notably the reaction temperature, pH, concentration of the sialic acid donor (cGMP) and concentration of the

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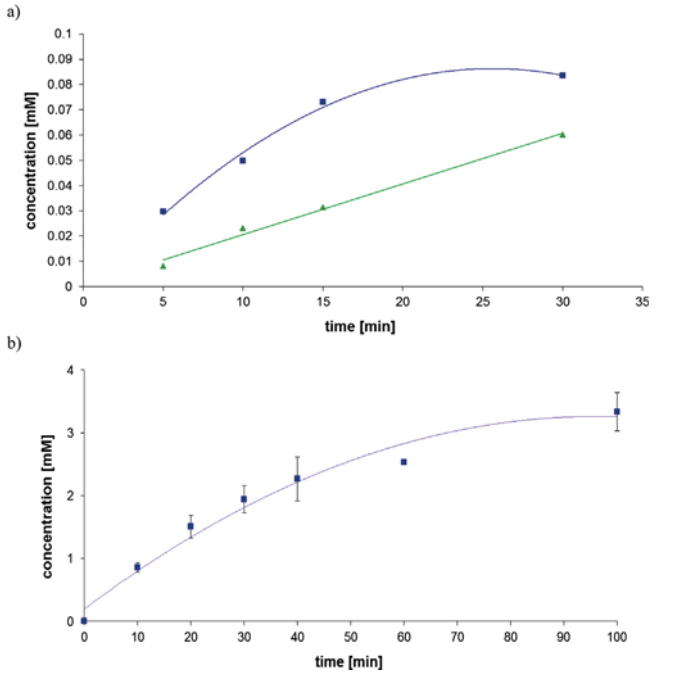

Fig. 2. (a) Accumulation of 3′-sialyllactose and sialic acid over time (25 min) at low concentration of lactose: 36.8 mM lactose, at 25 °C, pH 5.5, and 2.6 mM sialic acid contained in cGMP; measured by HPAEC-PAD; 3′-sialyllactose; sialic acid. (b) Accumulation of 3′-sialyllactose over time at high lactose, 117 mM lactose, 25 °C, pH 5.5, and 4.6 mM sialic acid bound in cGMP; measured by LC/MS. No free sialic acid was detected.

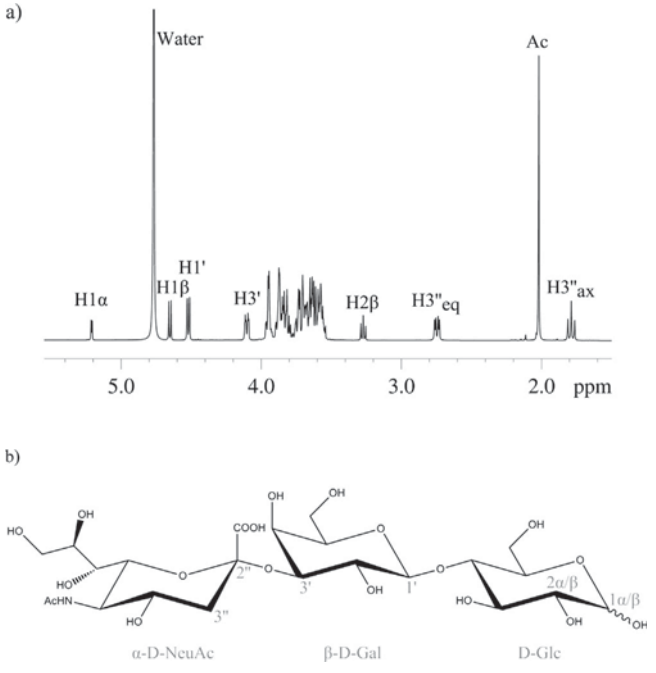

Fig. 3. (a) ^{1}H NMR spectrum of 3′-sialyllactose in D_2O with assignments of distinctive and discriminative resonances. The spectral region between 3.5 and 4 ppm holds the remaining proton resonances for 3′-sialyllactose and besides a contamination with formate the purity of the sample is very high. ^{1}H NMR and ^{13}C NMR assignment data of 3′-sialyllactose are given in Supplementary material S4. (b) Structure of 3′-sialyllactose with selected atom numberings used in the NMR assignment.

acceptor (lactose), revealed that increased temperature (from 15 to 25 °C) and decreased pH (from 7.5 to 5.5) gave rise to enhanced yields of 3′-sialyllactose (Supplementary material, Figure S2), but at the reaction optimum at pH 5.5 and 25 °C, at low concentration of the acceptor, lactose (4 mM), only low levels of 3′-sialyllactose were produced, and free sialic acid was released simultaneously (Fig. 2a). These data signified that the reaction conditions did not sufficiently suppress the sialidase activity of the enzyme. The retainment of some sialidase activity was, however, in agreement with previous results reported for *T. rangeli* mutants expressed in *E. coli* [17]. Nevertheless, when higher lactose concentrations (117–351 mM) were used, i.e. in effect a higher substrate acceptor:donor ration, the Tr6 enzyme was able to catalyze the production of high amounts of 3′-sialyllactose by *trans*-sialidase action without simultaneously catalyzing release of free sialic acid (Fig. 2b). At this high acceptor concentration, and higher acceptor:donor ratio, the specific activity of the Tr6 *trans*-sialidase activity was determined to be 16.2 μmol min^{-1} mg^{-1}.

3.3. Enzymatic production, purification and analysis of 3′-sialyllactose at 5 liter-scale

For the enzymatic production of 3′-sialyllactose 5.3 mg/L of Tr6 was incubated for 20 min at the optimal reaction conditions of pH 5.5, 25 °C, using 117 mM lactose (equivalent to 40 g/L) and 4.6 mM sialic acid residues bound in cGMP (equivalent to 26 g cGMP/L). After deactivation of the enzyme, the 3′-sialyllactose product was purified via a two-step down-stream process: the first step was ultrafiltration to remove cGMP and Tr6. The permeate was then collected and after freeze-drying 180 g of dry product was obtained containing 3′-sialyllactose, unreacted lactose, sialic acid and buffer used in the enzymatic process. In the second down-stream processing step, the different compounds were then separated by anion exchange chromatography using ammonium formate as the eluent (Figure S3). The quality of the separation was confirmed by capillary LC/MS and NMR spectroscopy (as discussed below). In order to remove the ammonium formate, the samples were lyophilized six times. The final yield of 3′-sialyllactose was 3.6 g, and the amount of free sialic acid was 133 mg.

Only approximately 50% of the sialic acid residues in the cGMP are bound at the 3′-position, whereas the rest is bound at the 6′-position [23]. Since the Tr6 catalyzed the production of 3′-sialyllactose, it can be assumed that only 3′-bound sialyl, i.e. ~50% of the total sialic acid in cGMP, was available for this enzyme. Therefore, the molar yield of 3′-sialyllactose was ~50% [47.74%] based on the available 3′-sialic acid in cGMP.

The corresponding molar yield of the sialyllactose was 1% on the lactose, and for the free sialic acid the yield was ~2% based on the total sialyl-residues in the cGMP.

The identity of the 3′-sialyllactose product was confirmed by capillary LC/MS and NMR (Fig. 3). 3′-Sialyllactose obtained from the 5 L scale reaction eluted at the same time as the standard 3′-sialyllactose (data not shown), indicating that the structure of the compound produced was 3′-sialyllactose. Both 6′-sialyllactose and 3′-sialyllactose standards were fully resolved confirming the ability of the PGC column to provide good separation of different oligosaccharides and stereoisomers [24]. No 6′-sialyllactose was detected in the product profile, only 3′-sialyllactose.

The ^{1}H and ^{13}C NMR assignments obtained using 1D and 2D NMR (Fig. 3 and supplementary material Figure S4) agreed with literature values for 3′-sialyllactose [25,26]. Besides resonances of 3′-sialyllactose the ^{1}H NMR spectrum also showed a singlet resonance at 8.44 ppm (data not shown), which resulted from residual ammonium formate from the purification buffer.

3.4. Enzymatic production of sialylated lacto-N-tetraose and fuco-N-pentaoses

It was also demonstrated that the Tr6 was able to catalyze the sialylation of lacto-*N*-tetraose and fuco-*N*-pentaoses, albeit the

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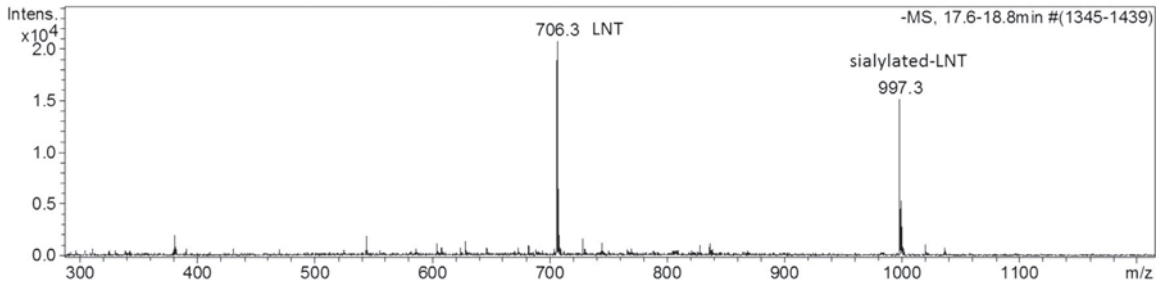

Fig. 4. Mass spectrum of ions eluted (at 17.6 and 18.8 min, respectively) in the post-reaction mixture of LNT incubated with cGMP and the Tr6 *trans*-sialidase: ion at *m*/*z* 706.3, LNT; ion at *m*/*z* 997.3, sialylated LNT.

yields of the products were quite low; hence 5–12 mg of sialylated LNT, LNnT, LNFP I and LNFP V were obtained employing the optimal reactions conditions for the Tr6 (pH 5.5, 25 °C), and as high as possible acceptor/donor ratio (see Section 2.7) (Figure S5). LC/MS analysis showed the molecular species of LNT and sialylated-LNT with the mass of sialylated-LNT at 997.3 (Fig. 4), corresponding to the sum of the LNT ion (706.3) and dehydrated sialic acid (291) [27]. The mass of sialylated LNnT was also 997.3, whereas the masses for sialylated LNFP I and LNFP V, respectively, were both determined to be 1143 in agreement with the mass increment of 146 produced by addition of fucose (data not shown). From the LC-MS results it was evident that the sialylated products of LNT, LNnT, LNFP I and LNFP V were predominantly singly sialylated (Figure S5).

4. Discussion

In the present study, the mutated *T. rangeli* sialidase, Tr6, was codon optimized and expressed at a relatively high yield in *P. pastoris* (1 g/L). In comparison, previously, only 5 mg/L of the *trans*-sialidase from *T. cruzi* was produced in the same expression host, *P. pastoris* [28]. This gene shared 67% homology to that of the wild type gene from *T. rangeli*. The relatively high yield of Tr6 in the present study could be due to the codon optimization or the more controlled conditions and higher cell density attained in a fermentor as compared to shake flasks. A high amount of Tr6 was furthermore a prerequisite for a careful examination of the *trans*-sialidase catalytic abilities of Tr6 at a range of process conditions examined in the present study. At the optimal process conditions it was observed, that the Tr6 sialidase from *T. rangeli*, could be tuned into an efficient *trans*-sialidase with more than 93% *trans*-sialidase activity and only 7% residual sialidase activity. These results differed from the *trans*-sialidase and sialidase activities recorded for a Tr6 enzyme produced in *E. coli*, which showed relatively lower *trans*-sialidase activity [17].

Whereas high substrate concentrations in general promote high catalytic rates according to Michaelis–Menten kinetics, high acceptor:donor ratios are advantageous for promoting *trans*-sialidase reactions, and generally used in *T. cruzi trans*-sialidase catalysis studies [e.g. 8, 29] as well as in β-galactosidase catalyzed galacto-oligosaccharide synthesis reactions [30,31]. When lactose is the acceptor for the *trans*-sialidase catalysis a high excess of acceptor is not an economic problem, but once more expensive tetra- and penta-saccharides are acceptors, measures such as recycling of the unused acceptor may have to be considered when comparing the application potential. Another issue is enzyme re-use to improve biocatalytic productivity. A time study performed at high lactose acceptor concentration was used in the present work to determine the maximal, specific *trans*-sialidase activity of Tr6. The specific Tr6 *trans*-sialidase activity was determined to 16.2 nmol min^{-1} per μg of pure protein (i.e. 16.2 μmol min^{-1} mg^{-1}). This specific activity was in essence equivalent to the apparent V_{max} of 14.7 nmol min^{-1} μg^{-1} reported for the similarly mutated *T. rangeli* sialidase expressed in *E. coli* with 3′-sialyllactose as donor and lactose as acceptor substrate [17], indicating that the maximal conversion rate found, was in fact equal to the maximum catalytic rate of the Tr6 enzyme. It is also interesting to note, that the specific Tr6 *trans*-sialidase activity (of 16.2 μmol min^{-1} mg^{-1}) was ~4 times higher than the reported V_{max} of the *T. cruzi trans*-sialidase (V_{max} 3.8 nmol min^{-1} μg^{-1}) measured using 3′-sialyllactose as the donor and lactose as the acceptor [17], and ~40,000 times higher than the maximal specific activity (V_{max} 0.41 nmol min^{-1} mg^{-1}) reported by Agusti et al. [32] for *T. cruzi trans*-sialidase catalyzed transfer of sialyl from 3′-sialyllactose to terminal galactose in (synthetic) Gal*p*(β1 → 6)GlcNAc mucins of the type involved in infection and pathogenesis of *T. cruzi*. With cGMP as the donor (at 5 g/L) and lactose as the acceptor molecule (at 20 g/L) the rate of sialyllactose synthesis using the *trans*-sialidase from *T. cruzi* occurs at a rate which is ~450 times less than that obtained in the present study with Tr6 [14]. As well, *Bifidobacterium infantis* sialidase catalyzed *trans*-sialylation, employing high levels of both cGMP and lactose [15], producing only a low level of sialyllactose, took place at a rate which was 87-fold lower than that obtained for Tr6 in the present study.

However, the specific V_{max} rates reported recently for optimal galactose transfer reactions for galacto-oligosaccharide synthesis using sucrose as donor and lactose as acceptor, employing the widely studied *Bacillus circulans* β-galactosidase (commercially known as Biolacta FN5′) may reach ~2 mM min^{-1} [31]. This specific activity is ~12 times higher than the specific *trans*-sialidase activity of Tr6 of 16.2 nmol·min^{-1} μg^{-1} assuming an enzyme dosing of the Biolacta FN5′ based on a *para*-nitrophenol galactoside activity of the *B. circulans* β-galactosidase of ~1 U/mg [31,33].

In general it has been shown that a few hydrolases can carry out a reverse reaction to synthesize organic molecules when the enzymatic process conditions have been optimized. This optimization can be performed by changing the reaction conditions such as temperature in the case of β-galactosidase for galactooligosaccharide production [34], glucose content in the case of the acyltransferase for production of glucose emulsifiers [35], and maltose level in the case of generating isomaltooligosaccharides (IMO) by glucoamylase [36]. These studies also showed that optimized process conditions can have a profound, stimulating effect on the rate of production and final yield of the products. The latter is in agreement with the present work whereby a sialidase with low *trans*-sialidase has been tuned by favorable process conditions to exert a very high *trans*-sialidase activity. The level of Tr6 produced in *P. pastoris* allowed a scaling of the enzymatic process to 5 L to establish proof-of-concept for the Tr6 *trans*-sialidase productivity at scale, providing 3.6 g of 3′-sialyllactose. To further validate the in vitro concept with the new biocatalyst, LNT, LNnT, LNFP I and LNFP V were also successfully decorated with sialic acid derived from cGMP using the same procedure as employed for 3′-sialyllactose production. The availability of optimized enzymes able to act on

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components from large scale side-streams from the agricultural and dairy industries is a very important first part of the decision base for the food industry for developing competitive processes for new functional food ingredients. The further interplay between molecular evolution of the biocatalyst, i.e. Tr6, and biochemical engineering optimization could further increase the yield of the process developed here.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.procbio.2013.10.023.

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10.2 Rational design of a new *Trypanosoma rangeli* trans-sialidase for efficient sialylation of glycans

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Rational Design of a New *Trypanosoma rangeli* Trans-Sialidase for Efficient Sialylation of Glycans

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Abstract

This paper reports rational engineering of *Trypanosoma rangeli* sialidase to develop an effective enzyme for a potentially important type of reactivity: production of sialylated prebiotic glycans. The *Trypanosoma cruzi* trans-sialidase and the homologous *T. rangeli* sialidase has previously been used to investigate the structural requirements for trans-sialidase activity. We observed that the *T. cruzi* trans-sialidase has a seven-amino-acid motif (197–203) at the border of the substrate binding cleft. The motif differs substantially in chemical properties and substitution probability from the homologous sialidase, and we hypothesised that this motif is important for trans-sialidase activity. The 197–203 motif is strongly positively charged with a marked change in hydrogen bond donor capacity as compared to the sialidase. To investigate the role of this motif, we expressed and characterised a *T. rangeli* sialidase mutant, Tr13. Conditions for efficient trans-sialylation were determined, and Tr13's acceptor specificity demonstrated promiscuity with respect to the acceptor molecule enabling sialylation of glycans containing terminal galactose and glucose and even monomers of glucose and fucose. Sialic acid is important in association with human milk oligosaccharides, and Tr13 was shown to sialylate a number of established and potential prebiotics. Initial evaluation of prebiotic potential using pure cultures demonstrated, albeit not selectively, growth of Bifidobacteria. Since the 197–203 motif stands out in the native trans-sialidase, is markedly different from the wild-type sialidase compared to previous mutants, and is shown here to confer efficient and broad trans-sialidase activity, we suggest that this motif can serve as a framework for future optimization of trans-sialylation towards prebiotic production.

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Competing Interests: The authors have filed a patent application ("A mutant sialidase having trans-sialidase activity for use in production of sialylated glycans", European Patent Application No. 13163551.8). This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in our guide for authors. All material relating to the patent is available in connection with this submission and the present article was used as the framework to design the patent application.

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Introduction

For production of human milk oligosaccharides (HMOs), glycan sialylation can be achieved chemically as well as enzymatically [1]. To achieve enzymatic synthesis, a trans-sialidase (TcTS) derived from *T. cruzi*, the causative agent of Chagas disease, has previously proven useful by transferring sialic acid from a donor to an acceptor glycan [2]. However, for industrial production of food-grade HMOs, it is a drawback that the enzyme constitutes an important virulence factor within *T. cruzi* [3]. Redesigning mutants of the non-pathogenic *T. rangeli* sialidase (TrSA) that possesses relatively low trans-sialidase activity [4] provides an attractive alternative for application in bioconversion processes. TrSA is 70% identical to TcTS and has the same overall tertiary structure. Both enzymes have been extensively characterised by biochemical, mutational and structural studies, and they share a common double displacement mechanism with a tyrosine as the catalytic nucleophile [5,6].

In TcTS, the acceptor binding site consists of Y119 and W312 which form stacking interactions with the acceptor sugar [7]. In TrSA, W313 (corresponds to W312 in TcTS) is found in a different conformation due to a Q284P substitution, while the Y120 (corresponds to Y119 in TcTS) is replaced by serine [8]. In addition to these differences in the acceptor binding site, a conserved D97 hydrogen bonds differently to sialic acid in the two enzymes, possibly due to the substitutions V96M and P98A. Correction of both the acceptor-binding site (S120Y, G249Y, and Q284P) and the sialic acid binding pocket (M96V, and A98P) is required to confer trans-sialidase activity (1% of TcTS activity) to TrSA, and the additional single mutations I37L (in this study named Tr6) and G342A further increase activity to 10% of the TcTS activity [9,4]. Kinetic data, however, indicate that the mutants display a >25-fold lower affinity for lactose and >100-fold higher k_{cat} for the undesired, competing hydrolysis [4], indicating ample room for further improvement.

In this study, we carried out a rational design of the *T. rangeli* mutant sialidase Tr6 based on the identification of a major difference (the motif constituted by amino acids 197–203) close to the binding cleft. We hypothesised that this motif might affect the trans-sialidase *vs.* hydrolase activity, notably due to the extremely

unusual (and substitution-wise unlikely) +3 charge change in this segment of the native trans-sialidase. Our production and characterisation of a *T. rangeli* mutant sialidase (Tr13) from the Tr6 parent incorporating this motif confirmed this hypothesis, since a reduced hydrolytic activity was observed which promoted overall trans-sialylation efficiency. Using a side-stream component from the dairy industry, casein glycomacropeptide (cGMP), as sialic acid donor for Tr13, we were able to sialylate a number of established and potential prebiotic glycans, demonstrating a broad acceptor specificity of Tr13.

Materials and Methods

Substrates

para-Nitrophenyl neuraminic acid (pNP-Neu5Ac) was purchased from Sigma-Aldrich (Steinheim, Germany). 3′-sialyllactose and 6′-sialyllactose were obtained from Carbosynth (Compton, United Kingdom). The commercial casein glycomacropeptide (cGMP) product LACPRODAN CGMP-20 with a sialic acid content of about 9% (w/w) was supplied by Arla Foods (Viby, Denmark), and low molecular weight impurities were removed by filtration with a 5 kDa membrane. 4-methylumbelliferyl-β-D-galactopyranoside (MU-Gal), β-lactose, lactulose, melibiose, maltose, and fucose were purchased from Sigma-Aldrich (Steinheim, Germany). Galactooligosacchardies (GOS) were purchased from Gulio Gross (Trezzano, Italy). Iso-maltooligosaccharides (IMO) were kindly provided by Taka Hayashi from Kyoto University, RISH (Gokasho, Japan).

Rational mutant selection

Sialidase catalytic domains were identified using NCBI Conserved Domain Search [10]. Pymol v1.3 (Schrödinger) was used to identify amino acids within 14 Å of the sialic acid binding site, based on the crystal structure with PDB code 1WCS, and was also used for preparing the figures. The *T. rangeli* sialidase mutant Tr6 (see below) and trans-sialidases from *T. cruzi* (TcTS) (Uniprot ID Q26966), *Trypanosoma congolense* (Uniprot ID G0WJG3) [11] and *Trypanosoma brucei* (Uniprot ID Q57XJ2) [12] were aligned using ClustalW [13]. Ranking of chemical difference between substituted amino acids in Tr6 *vs.* TcTS was done based on being first- or second sphere relative to the substrate and based on the polar/nonpolar and small/large distinction; such property-based selection turned out to correlate well with standard substitution matrices (BLOSUM62), i.e. the most unlikely substitutions were considered noteworthy. A comparison of loop structures in *T. cruzi* and *T. rangeli* with and without a substrate analogue DANA (2, 3-Dehydro-2-deoxy-N-acetylneuraminic acid) bound was made using structures of TcTS (PDB codes 1MS1 and 1MS3) and TrSA (PDB codes 1N1S and 1N1T). A 3D-model of Tr13 was made using the Modeller software [14] with 1WCS as the template.

DNA manipulations and strain construction

A gene encoding Tr6, *T. rangeli* sialidase mutant $TrSA_{5mut}$ (the sequence was extracted from PDB file 1WCS) with an additional mutation I37L [4], was codon-optimized for expression in *Pichia pastoris* and synthesized by DNA2.0 (Menlo Park, CA, USA). The synthesized gene was inserted in the *P. pastoris* expression vector pPICZαC (Invitrogen) between the *Xho*I and *Xba*I restriction sites generating a translational fusion to the α-factor signal sequence as well as a C-terminal *c-myc* epitope and a 6xHis tag (Figure S1). The plasmid was propagated in *Escherichia coli* NM522 grown at 37°C with shaking in low salt LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) supplemented with 25 μg/mL zeocin. This vector, pPICZα-Tr6, was used as a template for introduction of additional mutations by PCR using overlapping primers (Table 1). The PCR products were inserted in pPICZαC between the *Xho*I and *Xba*I sites. Constructs were sequenced to confirm the mutations and to assure that no unwanted mutations had been introduced by PCR. In the following, the mutants are denoted by the amino acid change compared to the parent (e.g. Tr6 Q123R), except for the multi-mutant denoted Tr13, in which amino acids 197–203 were changed from IADMGGR to VTNKKKQ. *P. pastoris* X-33 was transformed with the constructs by electroporation following the manufacturer's instructions (Invitrogen).

Protein synthesis and purification

For small-scale protein synthesis, *P. pastoris* X-33 strains harboring pPICZα with mutated genes were grown for three days in 180 mL BMMY (10 g/L yeast extract, 20 g/L peptone, 100 mM potassium phosphate (pH 6), 13.4 g/L yeast nitrogen base, 0.4 mg/L biotin and 0.5% methanol) shaking at 30°C. Protein synthesis was induced every 24 hours by addition of methanol to a final concentration of 0.5%. Cells were removed by centrifugation for 5 min at 3000 g and the supernatant was subsequently sterile filtered using a 0.2 μM Minisart filter (Sartorius AG). The supernatant was concentrated about 100-fold using Vivaspin20 concentrators with a 30 kDa cutoff (Sartorius AG). The 6xHis-tagged protein was purified from concentrated samples using Ni-sepharose (GE Healthcare) columns in accordance with manufacturer's instructions, followed by desalting on PD-10 columns (GE Healthcare) using 20 mM sodium phosphate buffer (pH 7.4), containing100 mM NaCl, and 10% glycerol. The protein sample was finally concentrated to about 200 μL using a Vivaspin0.5 concentrator with a 50 kDa cutoff (Sartorius AG).

For large-scale production, Tr6, Tr13, and Tr6 D363E were produced in a 5 L Sartorius Biostat Aplus fermentor as described previously [15]. The enzymes were purified by Cu^{2+} affinity column chromatography as described previously [16]. Protein concentrations were determined using the BCA protein assay (Thermo Scientific) with bovine serum albumin as standard.

Sialidase activity assays

Sialidase activity was measured in a reaction containing 50 mM phosphate-citrate buffer (pH 7), 0.75 mM pNP-NeuAc, and 3 μg/mL sialidase enzyme. The reactions were initiated by addition of substrate and were then followed spectrophotometrically at 410 nm at 30°C. pH 7 was chosen to enable detection of released pNP in a continuous assay. Reaction rates were normalized as percent of the activity of Tr6. For measurement of hydrolysis of natural substrates, the assay was done with either 1 mM 3′-sialyllactose, 1 mM 6′-sialyllactose, or 1 mM cGMP-bound sialic acid in 50 mM phosphate-citrate buffer (pH 5) using 1 μg/mL enzyme. Reactions were started by addition of enzyme and stopped by adding H_2SO_4 to a final concentration of 45 mM. Quantification of free sialic acid was done using a 2-thiobarbituric acid assay [17] with the modification that butanol extraction was substituted with mixing with dimethyl sulfoxide (DMSO) [18].

Trans–sialidase activity assay

Trans-sialidase activity was assayed as described previously [19] but with several modifications. Reactions were done in 50 mM phosphate-citrate (pH 6) at 30°C using 2.9 μg/mL enzyme. As donor substrate, 1 mM cGMP-bound sialic acid was used, and MU-Gal was used as acceptor. The low solubility in aqueous solution prevented the use of higher concentrations of MU-Gal than 0.5 mM. A solution of 87 mM MU-Gal in DMSO was diluted to 2 mM in 50 mM phosphate-citrate buffer (pH 6)

Table 1. List of primers.

Name	Sequence	Description
Tr_fwd	GCTCTCGAGAAGAGAGAGGCTGAAG	*Xho*I, Tr 5′
Tr_rev	CGCTCTAGAAATGCTGCTGTACCAGC	*Xba*I, Tr 3′
Q123S_F	CTATTGGACC**TCT**CACAGAGATGGATCTGACTGG	Q123S
Q123S_R	CATCTCTGTG**AGA**GGTCCAATAGTTCCTTGTCTTG	Q123S
R125G_F	GACCCAGCAC**GGA**GATGGATCTGACTGGGAACC	R125G
R125G_R	CAGATCCATC**TCC**GTGCTGGGTCCAATAGTTCC	R125G
G127A_F	GCACAGAGAT**GCT**TCTGACTGGGAACCATTGTTG	G127A
G127A_R	CCCAGTCAGA**AGC**ATCTCTGTGCTGGGTCCAATAG	G127A
E175Q_F	ACTTACTAAG**CAG**TTCGTAGGTGGAGTAGGCG	E175Q
E175Q_R	CTCCACCTACGAA**CTG**CTTAGTAAGTATGCCGTCGAACTC	E175Q
V177L_F	TAAGGAATTC**TTG**GGTGGAGTAGGCGCCG	V177L
V177L_R	CCTACTCCACC**CAA**GAATTCCTTAGTAAGTATGCCGTCG	V177L
V180A_F	CGTAGGTGGA**GCT**GGCGCCGCCATCGTG	V180A
V180A_R	TGGCGGCGCC**AGC**TCCACCTACGAATTCCTTAGTAAG	V180A
G202K_F	TGCTGACATG**AAG**GGAAGAGTATTTACAAAAATTATGTATTCC	G202K
G202K_R	ATACTCTTCC**CTT**CATGTCAGCAATTTGCACAG	G202K
N250R_F	AGTCGATTAC**AGA**AGACGTCTGGTGTACGAATCC	N250R
N250R_R	CCAGACGTCT**TCT**GTAATCGACTCGGTTATTAATGATTAGC	N250R
D363E_F	GAGATTAATACTAAT**GAG**GTTTATTCTCTTGTTTTTGTCCG	D363E
D363E_R	CAAGAGAATAAAC**CTC**ATTAGTATTAATCTCATGTAGGGAATATAATTTATC	D363E
13MUT_F	CCCTGTGCAA**GTAACTAATAAGAAGAAGCAA**GTATTTACAAAAATTATGTATTCCGAGG	13MUT
13MUT_R	TTGTAAATAC**TTGCTTCTTCTTATTAGTTAC**TTGCACAGGGTATACCAAATTAC	13MUT
P98A_F	GGTTGTCGAT**GCT**ACGGTCATAGTAAAGGGAAATAAGTTG	P98A
P98A_R	CTATGACCGT**AGC**ATCGACAACCCTTGAAACTG	P98A
Y249G_F	CCGAGTCGAT**GGA**AATAGACGTCTGGTGTACGAATC	Y249G
Y249G_R	GACGTCTATT**TCC**ATCGACTCGGTTATTAATGATTAGC	Y249G

Restriction sites are underlined and mutated nucleotides in bold.
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immediately before preparing the reactions. When crude enzyme preparations from *P. pastoris* were used, a background signal likely related to cleavage of MU-Gal by endogenous β-galactosidase was present. This background signal could be removed by washing the column eight times with 440 µL of 5 mM HCl after sample application without desorbtion of the sialylated product and this was therefore done routinely.

Optimisation of conditions for trans-sialylation

To determine the optimal conditions (pH, temperature, and concentration of donor and acceptor), a quadratic central composite design was used. MODDE Version 7.0.0.1 (Umetrics AB, Umeå, Sweden) was used as a tool to design the experimental frame and to fit and analyse the data by multiple linear regression analysis. The pH regimes 3, 4 and 5, incubation temperatures 20, 40 and 60°C, and concentrations of the acceptor lactose of 117, 234 and 351 mM were tested. Reactions were done using a fixed concentration of cGMP-bound sialic acid of 8 mM in 15 mM phosphate-citrate buffer with specified pH values, and using 15 µg/mL of Tr13 enzyme. Lactose and cGMP were solubilised in buffer and preincubated at specific temperatures, before the reactions were initiated by addition of enzyme. The biocatalysis process was allowed to proceed for 20 min before the reaction was stopped by heating for 10 min at 90°C. The concentration of sialyllactose was determined by HPAEC as described below.

For a time study of the trans-sialylation reaction, Tr13 was incubated as described above at pH 3 with 351 mM lactose, 8 mM cGMP-bound sialic acid and a reaction temperature of 25°C. The reaction was followed by sampling over a 100 min period, and the concentration of sialyllactose was determined by LC/MS as described below. Measurement at zero time was done using heat-inactivated enzyme. Three replicates were made and each data series fitted to a second-order polynomial function. For each data series, the slope at time zero was used to calculate the specific activity and the standard deviation.

Enzymatic production and purification of sialylated glycans

The reactions were carried out in stirred glass bottles in reaction volumes of 50 mL for melibiose and maltose, 88 mL for fucose, 100 mL for lactulose, and 250 mL for GOS and IMO. The reaction was performed in 15 mM phosphate-citrate buffer (pH 3) with 351 mM sialic acid acceptor (GOS, IMO, lactulose, melibiose, maltose and fucose) and 8 mM cGMP-bound sialic acid at 25°C using 15 µg/mL enzyme. Prior to the reaction, the substrates were pre-incubated in the buffer. The reaction was carried out for 20 minutes and then stopped by enzyme inactivation by heating at 90°C for 10 minutes.

The reaction mixture was applied to a HiScale 50/20 (GE Healthcare) anion exchange chromatography column packed with

402 mL of Sepharose Q FF. The separation was done at ambient temperature with an ÄKTA purifier 100 work station equipped with a P-900 pump, UV-900 monitor, and Frac-950 fraction collector, all controlled by UNICORN software (GE Healthcare). The elution was monitored at 210 nm. Elution was performed at a flow rate of 70 mL/min. Before injection, the column was equilibrated with 5 column volumes (CV) of water. After injection, the column was washed with 3 CV of water, followed by elution with 3.5 CV of 40 mM ammonium formate and subsequently with 2 CV of 400 mM ammonium formate for cleaning the column. After elution, the column was regenerated with 3 CV of water. Fractions of interest were collected automatically. The products were lyophilized and ammonium formate was removed by repeated solubilization and lyophilization. Product structures were determined by LC/MS, as described below.

High-performance anion exchange chromatography (HPAEC-PAD)

Quantification of sialyllactose was carried out by HPAEC-PAD analysis using a Dionex BioLC system consisting of GS50 gradient pumps, ED50 electrochemical detector, AS50 chromatography compartment coupled to an AS50 autosampler (Dionex Corp., Sunnyvale, CA). Samples (10 µL) were injected on a CarboPac™ PA1 (4 mm×250 mm) analytical column (Dionex Corp., Sunnyvale, CA) at a flow rate of 1 mL/min. The elution program was based on the method described in [20] except for the modifications in the eluent system given below. The eluent system comprised of deionised water (A), 0.5 M NaOH (B), 1 M NaOAc (C). For the first 3 min an isocratic elution of 80: 20 (% A:B) was applied, which was followed by a linear gradient from 80:20 (% A:B) to 60:20:20 (% A:B:C) from 3 to 27 min. Strongly retained anions were removed from the column by isocratic elution at 40:20:40 (% A:B:C) from 27 to 31 min. Subsequently, the column was re-equilibrated for 7 min with 80:20 (%A:B).

Capillary Liquid Chromatography/Mass spectrometry

For liquid chromatography/Mass spectrometry (LC/MS) analysis, an Agilent 1100 LC/Agilent 6340 ion trap MS system was used. Oligosaccharides were separated using a Hypercarb porous graphitic carbon (PGC) column (0.32×150 mm, 5 µm, Thermo scientific) at 30°C. Samples (0.5 µL) were loaded onto the column in 10 mM ammonium bicarbonate. Gradient elution was achieved using a binary solvent system consisting of (A) 10 mM ammonium bicarbonate, adjusted to pH 8.5 with ammonium hydroxide, and (B) 100% acetonitrile at a flow rate of 5 µL/min. The gradient was initially at 98:2 (% A:B) for 5 min, followed by a linear increase to 42:58 (% A:B) at 33 min. This concentration of B was held for 3 min. Subsequently the eluent was returned to 98:2 (% A:B) at 40 min and the system was allowed to equilibrate for 10 min prior to the next injection. All solvents used were of the highest HPLC grade. The mass spectrometry was performed in negative ion mode, and was scanned in the range m/z 150–2200 (2 microscans, maximum accumulation time of 150 ms, an ion current count of 200,000) followed by data-dependent MS2 scans of the four most abundant ions in each MS1 scan.

Bacterial growth assays on sialylated glycans

For testing growth of bacterial strains on sialylated glycans the following strains were used: *Bifidobacterium longum longum* (Danisco Global Culture Collection DGCC 232), *Bifidobacterium longum infantis* (DGCC 233), *Bifidobacterium longum infantis* (DGCC 1497), *Bifidobacterium longum infantis* (DGCC 2238), *Lactobacillus acidophilus* (NCFM, ATCC 700396), *Bifidobacterium longum* (Bl-05, DGCC 9917), *Bifidobacterium lactis* (HN019, DGCC2013), and *Clostridium perfringens* (ATCC 13124). Galactan from potato (Megazyme International LTD, Bray, Co. Wicklow, Ireland) was used as an established prebiotic standard control. The substrates were dissolved in water at 10% (w/v) and sterilized by sterile filtration (0.2 µm Minisart, Sartorius AG, Göttingen, Germany) except for a control substrate, galactan from potato (Megazyme International LTD, Bray, Co. Wicklow, Ireland), that due to its high viscosity was sterilised by UV-radiation for 30 seconds. The strains were precultured in MRS^- medium (de Man, Rogosa and Sharpe medium without glucose) with no additional sugars added for 24 h at 37°C under anaerobic conditions before being diluted with fresh MRS^- medium to 1% (v/v). Growth on test substrates was done by adding 20 µL of 10% test substrates and 180 µL 1% cell suspension in multiwell plates and growth was followed by measurement of optical density at 600 nm (OD_{600}) using Biolink® software (Labsystems) in a Bioscreen® C system (Labsystems, Helsinki, Finland) as described previously [21]. The growth in MRS^- medium without addition of carbohydrates was used as control. The experiments were done in three replicates for each strain, and carbohydrate substrate and growth was determined as the area under the growth curve. Data are given as mean values ± standard error.

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey-Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Weseley, Reading, MA).

Results and Discussion

Rational design of the *T. rangeli* sialidase mutant Tr13

In order to identify mutations likely to affect enzyme activity, we used two initial criteria: First, we considered only amino acids within 14 Å of the sialic acid-binding site. Secondly, based on an alignment between Tr6 and the efficient trans-sialidase TcTS, we considered the chemical difference between the amino acids, assuming that larger chemical differences, correlating with lower probability of substitution by random evolution, would be the most likely candidates for conferring increased trans-sialidase activity and/or reduce unwanted hydrolysis. We also inspected the impact of changes in surface exposure, hydrogen bonding, and the distance from the acceptor binding site. A sequence alignment with amino acids within 14 Å of the sialic acid-binding site and sites selected for mutagenesis are shown in Figure 1.

Among significant differences, the motif composed of amino acids 197–203 is IADMGGR in TrSA and Tr6, but is substituted to VTNKKKQ in TcTS. These substitutions cause a change in net charge of +3, and the MGGR → KKKQ sequence of substitution is highly unlikely to occur randomly (−4 in total from a BLOSUM62 matrix [22]). The substitution of two Gly residues to Lys could alter the rigidity of the loop. This changed rigidity might affect exclusion of water from the active site which could in turn explain the reduced hydrolytic activity observed in TcTS. To investigate this hypothesis, we compared the loops in structures of TcTS and TrSA with and without a ligand bound (Figure 2). A comparison of the loops shows little difference in the backbone position between the two enzymes and neither of them appeared to change conformation upon ligand binding. This indicates that the chemical properties of the side chains, rather than the size and backbone dynamics of the loops, are likely to determine differences in catalytic activity. Consequently, an alternative explanation for the role of the VTNKKKQ motif was sought in the introduction of a +3 charge difference between the two loops. This charge difference is also seen in the less similar trans-sialidase from

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T.rangeli Tr6    TIRERVVHSFRLPTIVNVDGVMVAIADARYETSFDNSFIETAVKYSVDDGATWNTQIAIKN 86
T.cruzi TcTS     --TERVVHSFRLPALVNVDGVMVAIADARYETSNDNSLIDTVAKYSVDDGETWETQIAIKN 85
T.congolense     GTTMRTVHSYRIPSIVEVGGVLMCVGDARYITSTDYFFTDTVAAYSTDGGRTWKREVIIPN 160
T.brucei         ----RTVHSFRIPSFVEVDGVLMGIGDARYLTSTDYFFTDTVAKYSADGGKTWKTEVIIEN 183

                           ▲                     * * *
T.rangeli Tr6    SRAS-SVSRVVDPTVIVKGNKLYILVGSFNKTRNYWTQHRDGS---DWEPLLVVGEVTKSA 143
T.cruzi TcTS     SRAS-SVSRVVDPTVIVKGNKLYVLVGSYNSSRSYWTSHGDAR---DWDILLAVGEVTKST 142
T.congolense     GRVDAHYSRVVDPTVVAKGNNIYVLVGRYNVTRGYWHNKNNRAGVADWEPFVYKGTVNVGT 221
T.brucei         GRVDPTYSRVVDPTVVAKADSVFVLVARYNVTKGYWHNENNAAGIADWEPFMYKGVVTKGA 244

                                       * *  *                ●●●●●●●
T.rangeli Tr6    ANGKTTATISWGKPVSLKPLFPAEFDGILTKEFVGGVGAAIVASNGNLVYPVQIADMGGRV 204
T.cruzi TcTS     AGGKITASIKWGSPVSLKEFFPAEMEGMHTNQFLGGAGVAIVASNGNLVYPVQVTNKKKQV 203
T.congolense     KDNATDVSISWER-TALKSLYNFPVSGSPGTQFLGGAGGGVVTSNGTIVLPVQARNKANRV 281
T.brucei         DGKTSDVRISWTK-TPLKPLYDFTVAGSKGTQFIGGAGNGVVTLNGTILFPVQARNEDNAV 304

                                                    ▲*
T.rangeli Tr6    FTKIMYSEDDGNTWKFAEGRSKFGCSEPAVLEWEGKLIINNRVDYN-------RRLVYESS 258
T.cruzi TcTS     FSKIFYSEDEGKTWKFGKGRSAFGCSEPVALEWEGKLIINTRVDYR-------RRLVYESS 257
T.congolense     VSMILYSADDGKSWHFGKGEAGVGTSEAALTEWDGKLLISARSDGG-----QGYRMIFESS 337
T.brucei         VSMVMYSVDDGVSWHFARGETALLTSEASLTEWNGKLLMSARTDTSGVNVEGGFRKVFESN 365


T.rangeli Tr6    DMGKTWVEALGTLSHVWTNSPTSNQP-------DCQSSFVAVTIEGKRVMLFTHPLNLKGR 312
T.cruzi TcTS     DMGNSWLEAVGTLSRVWGPSPKSNQP-------GSQSSFTAVTIEGMRVMLFTHPLNFKGR 311
T.congolense     DLGATWKEMLNSISRVIGNSPGRSGP-------GSSSGFITVTVEGVPVMLLTHPKNLKGS 391
T.brucei         NLGATWEESLGTISRVIGNSPDRTKPSPTANYPGSSGALITVTLGDVPVMLITHPKNTKGA 426

                                                          *
T.rangeli Tr6    WMRDRLHLWMTDNQRIFDVGQISIGDENSGYSSVLYKDD-KLYSLHEINTNDVYSLVFVRL 372
T.cruzi TcTS     WLRDRLNLWLTDNQRIYNVGQVSIGDENSAYSSVLYKDD-KLYCLHEINSNEVYSLVFARL 371
T.congolense     YYRDRLQMWMTDGNRMWHVGQVSEGDDNSAYSSLLYTPDGVLYCLHEQNIDEVYS------ 446
T.brucei         WSRDRLQLWMTDGNRMWLVGQISEGDDNSAYSSLLLARDGLLYCLHEQNIDEVYG------ 481
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Figure 1. Sequence alignment of sialidase catalytic domain from Tr6 and related trans-sialidases. Tr6 and trans-sialidases from *T. cruzi* (Uniprot ID Q26966), *T. congolense* (Uniprot ID G0WJG3) and *T. brucei* (Uniprot ID Q57XJ2) were aligned using ClustalW. Amino acids within 14 Å of sialic acid binding site are shown in bold. The seven amino acid motif is indicated with filled circles, reverting mutations are indicated with a triangle while other mutated sites are indicated with asterisks.
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T. congolense (ARNKANR, charge +3), but not in that of *T. brucei* (ARNEDNA, charge –1). We hypothesised that the charge difference and/or the substantial change towards hydrogen-bond donors could affect the affinity for lactose or reverse the water network in the substrate-binding cleft (or reduce water affinity, i.e. increase K_m for hydrolysis to favour competitive binding of lactose), all to the effect of reducing hydrolysis. Energetically, the free energy cost of aligning water molecules in the cleft (rearrangement free energy of ~6.3 kJ/mol [23]) should be lower than for fully excluding them (free energy of hydration of water into water with an upper cost of ~35 kJ/mol [24]).

A homology model of mutant Tr13 based on Tr6 with the additional seven mutations of the VTNKKKQ motif is shown in Figure 3. The mutations are relatively far, ~14 Å, from the acceptor binding site and therefore unlikely to affect acceptor binding directly. Water structure is from recent work known to be a combination of symmetric, directed tetrahedral structure and momentaneous asymmetry from breaking of hydrogen bonds [25]. The substantial increase of hydrogen bond donation from the loop side suggests that a tetrahedral water network of the symmetric type will be more likely to be inverted. This would change the electrostatic field in the cleft and potentially disrupt or even reverse the water network in the active site. Hydrolysis requires a water network aligned with oxygen lonepairs towards the sialic acid, whereas a strong positive charge and hydrogen-donor tendency at the edge of the binding cleft, as seen in the native trans-sialidase would work towards a partial reversal of such a network, turning oxygen lone pairs towards the field of the lysines and correspondingly impairing the nucleophilicity of the water network in the cleft. Such a disruption of the water network could be the explanation for TcTS's exquisite quenching of hydrolysis, not achieved by previous *T. rangeli* sialidase mutants [4].

In addition to the VTNKKKQ motif probed by Tr13, we also investigated several single-site mutations from Tr6, to understand the properties of previously described mutants in more detail. Two such mutations were reverting mutations of the parent, P98A and Y249G, while V180A was the mutant previously reported to at least partially reduce hydrolase activity [4]. As further candidates for improving trans-sialidase activity, several new single mutations were also made from Tr6: R125G, E175Q, G202K, N250R,

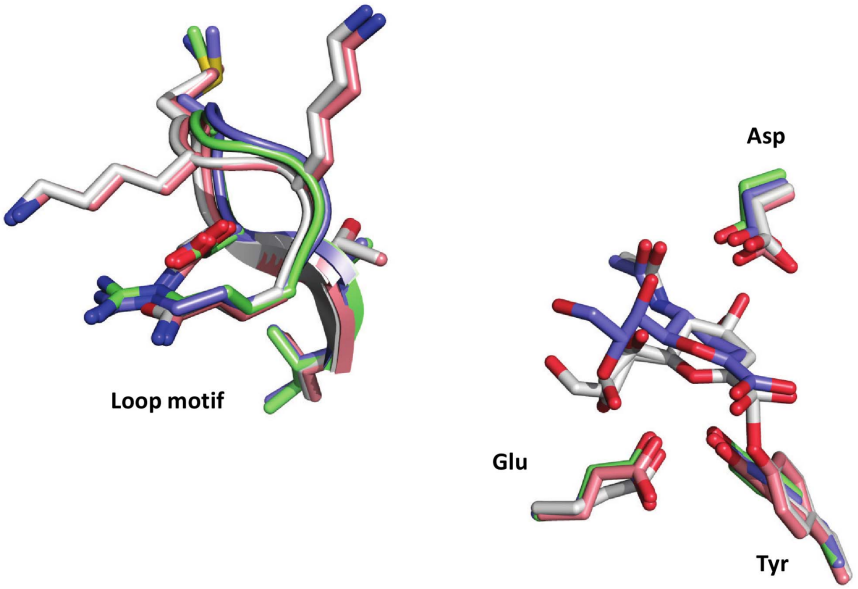

Figure 2. Comparison of the loop motif in TcTS and TrSA. Structures of TcTS (PDB 1MS1; grey and 1MS3; light red) and TrSA (PDB 1N1T; blue and 1N1S; green), with and without the sialic acid analogue DANA bound respectively, were compared. The loop motif constituted by amino acids 196–202/197–203 and DANA bound in active site between catalytic residues Glu-230/231, Tyr-342/343 and Asp59/60 in TcTS and TrSA respectively are displayed.
doi:10.1371/journal.pone.0083902.g002

Q123R, G127A, I177L, and D363E. These mutations were notable either by their positions close to the binding cleft and/or by conferring a chemical difference.

Sialidase mutant Tr13 displays increased trans-sialylation activity

To assess the performance of the mutants, we produced them in *P. pastoris* shake flask cultures and tested their trans-sialidase activity using a fluorescence-based assay using cGMP as sialic acid donor and methylumbelliferyl-β-D-galactopyranoside (MU-Gal) as acceptor (Figure S2). The enzyme amounts obtained were too low to allow a detailed characterization of the mutants. Since the detected maximum product yield will be a measure of both the trans-sialidase activity (product formation) and the hydrolase activity (donor and product degradation) of the enzyme, it was considered a relevant assay for an initial screen of the mutants.

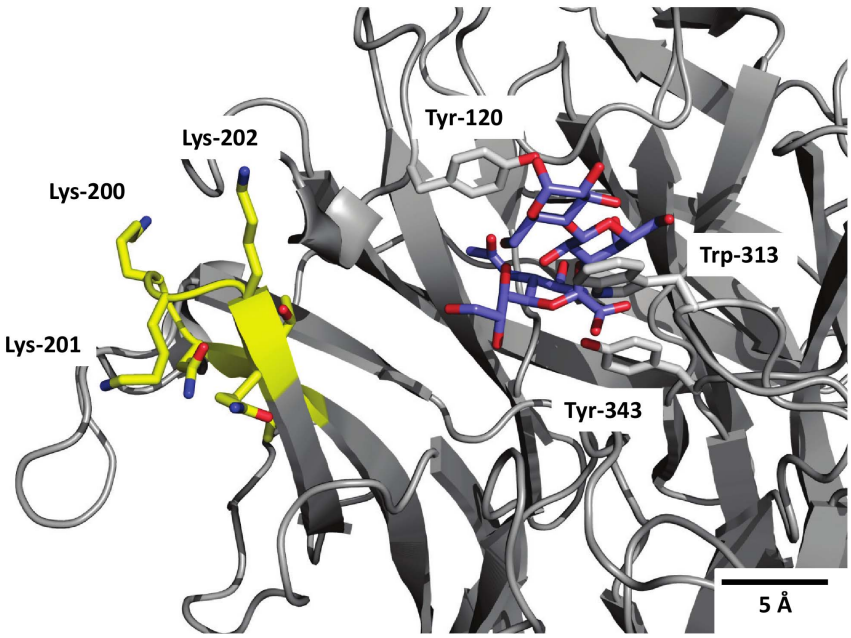

Figure 3. Homology model of Tr13. Close-up of active site with sialyllactose docked (blue). Acceptor binding site residues Tyr-120 and Trp-313 and catalytic nucleophile Tyr-343 side chains are shown in grey. The seven introduced amino acids are shown in yellow.
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Although all the mutants were active, only Tr13 and Tr6 D363E performed at a level comparable to Tr6 while all other mutants displayed a decreased trans-sialidase activity. These two mutants were therefore selected for further analysis, since the primary objective of this work was to generate mutants displaying improved trans-sialidase activity. It is worth noting that the single mutation G202K, which is part of the VTNKKKQ motif, reduced trans-sialidase activity. This indicates a co-operation effect of the amino acids of the motif and not an effect additively derived from the single-site mutations. For the reverting mutations P98A and Y249G it could be concluded that they improve but are not essential for trans-sialidase activity.

For further evaluation we produced the parent enzyme Tr6 and the two mutants Tr13 and Tr6 D363E in a 5 L fermentor. This allowed us to evaluate both hydrolase and trans-sialidase activity (Figure 4). Hydrolase activity was tested on the artificial substrate pNP-Neu5Ac as well as on the natural substrates 3′-sialyllactose, 6′-sialyllactose, and cGMP. None of the enzymes exhibited detectable activity on 6′-sialyllactose (data not shown) in turn indicating that the α-2,6-linked sialic acid in cGMP was not used as a sialyl donor when cGMP was employed as donor substrate for the *T. rangeli* mutants. The α-2,6-linked sialic acid constitutes about 50% of total sialic acid content in cGMP [26]. While both Tr13 and Tr6 D363E had a decreased activity on pNP-Neu5Ac, only Tr13 had a lower hydrolase activity on 3′-sialyllactose and cGMP (Figure 4A).

In the trans-sialidase activity assay, the initial reaction rate represents the trans-sialidase reaction rate, while maximum product formation is a measure of both trans-sialidase activity (product formation) and hydrolase activity (product degradation). Thus, Tr6 and Tr13 appeared to have similar trans-sialidase activities (as measured by their initial reaction rates), while Tr6 D363E had a slightly lower activity, but under the applied reaction conditions Tr13 accomplished twice the maximal yield (Figure 4B), underscoring the importance of the VTNKKKQ motif for total yields due to reduced hydrolysis activity on the product.

The improved maximal yield obtained for Tr13 catalysis suggests that the VTNKKKQ motif does not affect the acceptor binding affinity, but rather uniquely reduces the hydrolytic activity (water k_{cat} and/or K_m), probably by impairing water nucleophilicity for attack on sialic acid (partial reversal of the water network) and/or by reducing water's retention time in the active site in competition with the acceptor. The effect may be acceptor-dependent, as the total extent of hydrolysis not only depends on the impaired water network, but also the K_m of the acceptor during trans-sialylation, which affects acceptor *vs.* water retention time and thus, the competition between hydrolysis and trans-sialylation.

Of the three lysines introduced in Tr13, K200 and K201 are partly shielded from the active site (Figure 3) while K202 points towards the center of the site. Changing only K202 led to a reduced activity compared to the parent. The exact mechanism by which the mutations exert their effect remains unclear and certainly these mutations do not completely account for the exceptionally low hydrolase activity and the higher affinity for lactose in TcTS compared to Tr6. Tr13 however, represents a major step forward by providing the first-ever design of a markedly hydrolysis-impaired sialidase mutant. Within protein engineering at large, viable mutants with improved properties that deviate so substantially from a wild type (by 13 site changes including a 7-amino acid loop structure with a +3 charge difference) are unusual.

Optimization of reaction conditions for Tr13

Statistically designed experiments were performed to determine reaction conditions that favour the trans-sialidase activity of Tr13. The influence of the following factors was investigated: temperature (20–60°C), pH (pH 3–5) and concentration of a standard acceptor for this reaction, lactose (117–351 mM), while using a fixed concentration of cGMP (8 mM). Reactions were allowed to proceed for 20 min, and after terminating the reactions, the concentration of 3′-sialyllactose was determined by HPAEC. No product formation was observed in controls with heat-inactivated enzyme. The highest product yield was obtained at 351 mM lactose (highest tested), pH 3 (lowest tested) and at 20°C (lowest tested) (data not shown). A time study was performed at these conditions and the specific trans-sialidase activity of the enzyme was determined from this study shown in Figure 5. The specific trans-sialidase activity, measured as number of sialyl-moieties transferred, of Tr13 was 4.4 +/−0.7 nmol*min^{-1} per μg of enzyme on cGMP. It was apparent that for a higher product yield, the reaction time could be successfully extended from 20 to 100 minutes with no detectable product degradation, since no free sialic acid was detected by LC/MS. Although the maximum yield was not determined in the study, it could be concluded that at least

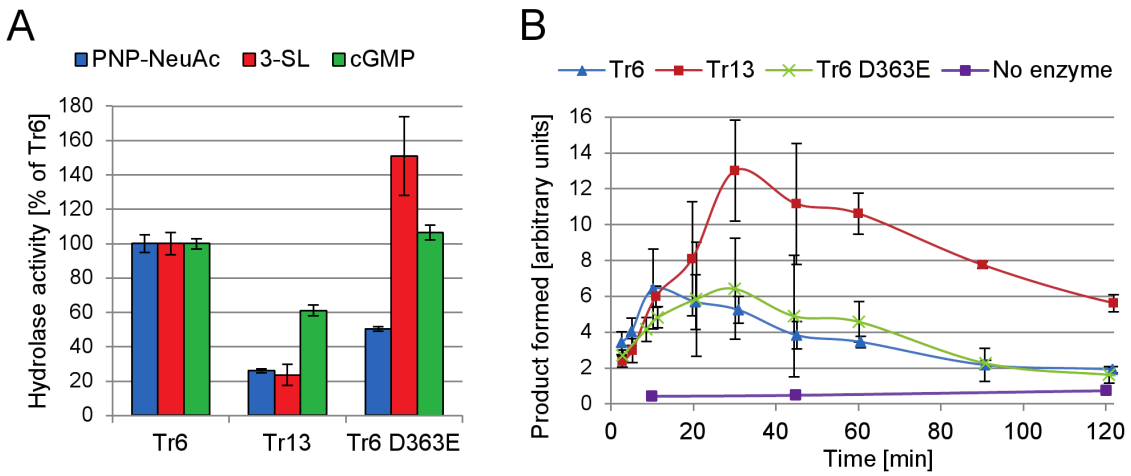

Figure 4. Enzyme activity of Tr6 and selected mutants Tr13 and Tr6 D363E. A) Hydrolase activity on substrates pNP-Neu5Ac, 3′-sialyllactose, and cGMP. B) Trans-sialidase activity using cGMP as sialic acid donor and MU-gal as acceptor.
doi:10.1371/journal.pone.0083902.g004

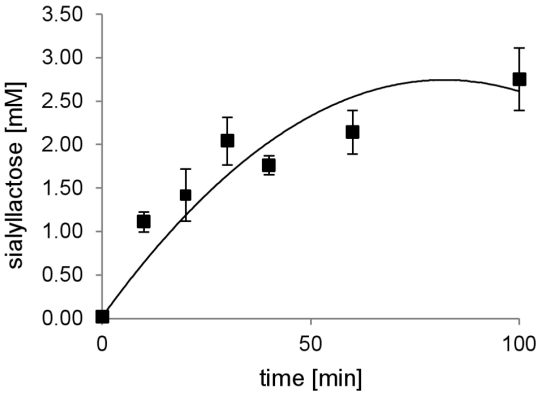

Figure 5. Time study of trans-sialylation catalysed by Tr13. Accumulation of 3′-sialyllactose over time in 25°C, pH 3, 351 mM lactose and 8 mM cGMP-bound sialic acid.
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~2.5 mM 3′-sialyllactose could be produced. In cGMP, α-2, 3- and α-2,6-bound sialic acid is in a ratio of about 1:1 [26], and hence only 4 of the 8 mM cGMP-bound sialic acid was theoretically accessible giving a yield of about 63%.

That the highest product yield was obtained at very low pH is surprising, since pH_{opt} for TcTS (using N-acetyllactosamine as acceptor) is pH 7 [27]. The introduction of charged residues in the vicinity of the catalytic residues might modulate the pH optimum but unlikely by such magnitude. A temperature optimum of 13°C has been reported for TcTS at low acceptor concentration (7.2 μM) due to increased affinity for the acceptor at low temperature while increasing acceptor concentration increased T_{opt} [28]. In our case, however, a very high acceptor concentration was used, indicating a different mechanism for the optimum at low temperature.

Sialylation of various prebiotic glycans revealed promiscuous acceptor substrate specificity of Tr13

We also examined the possibility of using Tr13 for synthesis of chimeric molecules, probing the possibility of combining the prebiotic effect with sialic acid as found in many HMO molecules to assess potential, enhanced beneficial properties. To this end, we sialylated the well-documented prebiotics GOS, IMO, and lactulose as well as three other compounds, melibiose, maltose, and fucose. This also provided a possibility to probe the acceptor substrate specificity of this enzyme. For TcTS it is generally accepted that terminal galactose moieties function as acceptor with lactose being a better acceptor than melibiose and galactose, glucose being a poor one [29]. Here we tested terminal galactose, terminal glucose, and glucose and fucose monomers as acceptor substrates for Tr13.

The sialylated oligosaccharides were enzymatically produced in phosphate-citrate buffer at pH 3 at ambient temperature. 351 mM sialic acid acceptor and 8 mM cGMP-bound sialic acid was used. After incubation for 20 minutes, the enzyme was heat-inactivated and the sialylated products were purified by anion exchange chromatography (Figure 6). In the unbound fractions, there was always neutral, unreacted acceptor, which was eluted with water. Applying 40 mM ammonium formate led to elution of negatively charged compounds, i.e. sialylated products and afterwards free sialic acid. According to LC/MS analysis, the sialylated compounds were completely separated from sialic acid as well as from the acceptor used in the reaction. The highest yields were achieved when fucose (1.5 mM) and lactulose (1.9 mM) were used as the sialic acid acceptors. Due to the relatively low yield compared to the concentration of acceptor substrates, it was relevant to assure that the substrates did not contain sialylated compounds. Analysis of the lactulose and melibiose preparations by LC/MS identified no contamination with sialylated compounds or sialic acid. The yields of sialylation products of GOS and IMO were lower. GOS and IMO preparations were a mixture of oligosaccharides of different chain length. The yields for each of the different chain lengths might have differed from the average since sialylation of smaller species appear to be favoured, at least for trans-sialylation with bacterial sialidases [30]. The composition of products of sialylation of GOS and IMO was complex (Table 2): Four and five different sialylated compounds, respectively, were obtained. In the case of GOS, the product of the lowest molecular weight was sialyllactose (m/z of 632), whereas incubation of IMO with cGMP led to production also of sialylated glucose (m/z of 470), since the starting material was abundant in that monomer.

Of the compounds produced, sialyllactulose was produced in the highest molar yield. The presence of galactose and the 1,4-β bond between galactose and fructose may make lactulose more accessible for Tr13, since for the similar-size acceptors melibiose (1,6-α-bound galactose) and maltose (1,4-α-bound glucose), yields were less than 60% of those obtained with lactulose. It is well established that glycans containing terminal galactose can be sialylated by TcTS, but the fact that Tr13 could also sialylate terminal glucose and even glucose and fucose monomers was surprising and indicated a high level of acceptor promiscuity. To our knowledge, this is the first report of such broad acceptor substrate specificity, and it can potentially be a valuable asset for enzymatic synthesis of a broad range of sialylated glycans using the Tr13 enzyme as catalyst.

Potential prebiotic effect of various sialylated glycans

The end-goal for this ongoing research effort is the enzymatic synthesis of novel functional food ingredients. As an initial assessment of potential prebiotic properties of the sialylated glycans, their growth promoting activity on selected bacterial pure cultures comprising three strains of *B. longum* subs. *infantis*, two *B. longum* subsp. *longum*, one *B. lactis*, one *L. acidophilus* and one pathogenic strain of *C. perfringens* was evaluated. This was done using a Bioscreen microtiter system, and the growth performance was expressed as area under the growth curve [21]. To assess the impact of sialylation it would have been relevant to compare with growth on the unsialylated acceptor molecules, but since the distribution of sialylated molecules of different chain length in case of GOS and IMO was not quantified, we decided to use galactan from potato as a control due to its confirmed prebiotic properties [31].

The data presented in Table 3 revealed that within the group of probiotic strains, sialylated melibiose and maltose did not appear to promote growth. For *B. infantis* 233, *B. infantis* 1497, and *B. longum* 232, growth was promoted by different sialylated compounds while sialylated fucose promoted growth for all three. In the case of *B. infantis* 2238, *B. lactis*, *L. acidophilus*, and *B. longum* 9917, none of the sialylated compounds promoted growth, while *L. acidophilus* grew well on the prebiotic control substrate galactan. *C. perfringens* grew significantly better than all the probiotic strains on the sialylated compounds, except on sialylmelibiose where growth was not tested due to only a limited amount of material being available.

It was assumed that a prerequisite for utilising the sialylated compounds would be the presence of a sialidase, as well as the ability to degrade the prebiotic backbone. All the *B. longum subsp.*

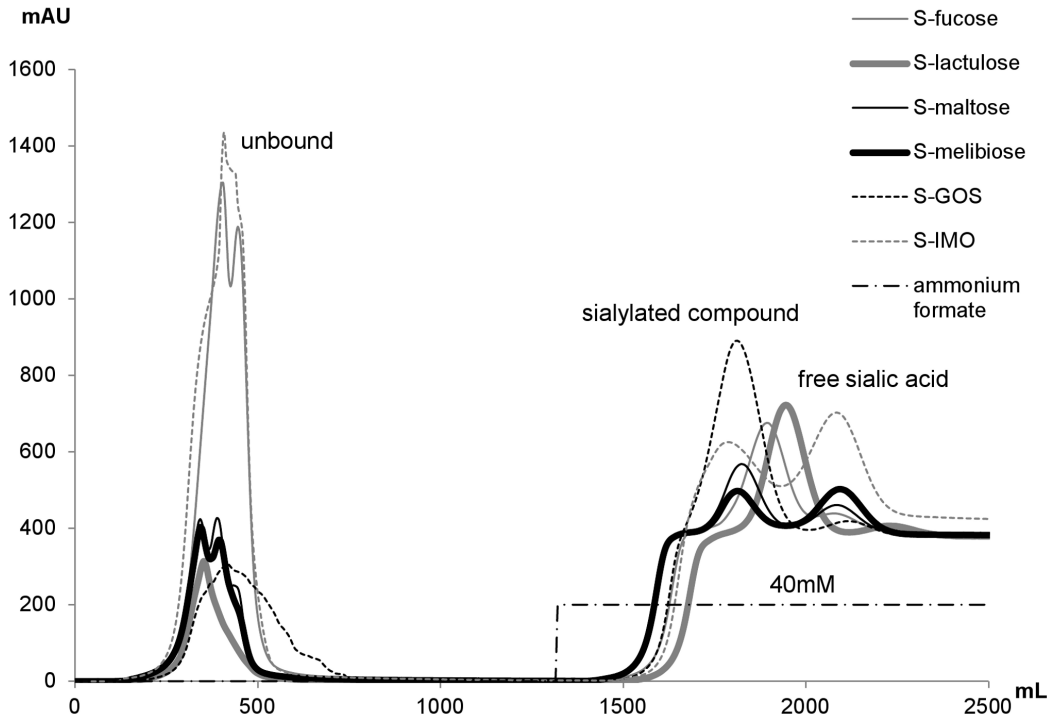

Figure 6. Anion exchange separation profiles for sialylated glycans. Sialylated glycans separated from sialic acid and unused acceptor separated by Sepharose Q and detected at 210 nm.
doi:10.1371/journal.pone.0083902.g006

infantis strains contained a sialidase (data not shown) as does *C. perfringens*, which contains the necessary enzymes for metabolising sialic acid [32]. Although variations in growth were found on different substrates, even within species, it was evident that the majority of the bacteria tested, including *C. perfringens*, to some extent were able to grow on the sialylated compounds. Recently, three fucosylated HMOs were shown to stimulate bifidobacteria, while *E. coli* and *C. perfringens* were unable to utilise the HMOs [33]. More interestingly, the organic acid fermentation product inhibited their growth, raising the possibility that this might also take place in a mixed culture with the molecules synthesised in this study. A mixed culture experiment taking into account bacterial interactions would be required to assess this. Furthermore, the potential functionality of these compounds as decoy molecules and

Table 2. Products of sialylation of various glycans analysed by LC/MS.

acceptor	m/z	product	Product conc.	Product yield	
	$[M-H]^-$		[g/L]	[w_{prod}/w_{accep}]	[mM]
GOS	632	SA-α-Gal-1,4-β-Glc	1.0	0.44%	ND
	794	SA-α-Gal-1,4-β-Gal-1,4-β-Glc			
	956	SA-α-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Glc			
	1118	SA-α-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Gal-1,4-β-Glc			
Fucose	454;	SA-α-Fuc	0.66	1.17%	1.46
Melibiose	632	SA-α-Gal-1,6-α-Glc	0.62	0.52%	0.98
Lactulose	632	SA-α-Gal-1,4-β-Fru	1.2	0.97%	1.84
Maltose	632	SA-α-Glc-1,4-α-Glc	0.66	0.55%	1.04
IMO	470	SA-α-Glc	0.72	0.60%	ND
	632	SA-α-Glc-α-Glc			
	794	SA-α-Glc-α-Glc-α-Glc			
	956	SA-α-Glc-α-Glc-α-Glc-α-Glc			
	1118	SA-α-Glc-α-Glc-α-Glc-α-Glc-α-Glc			

Yields are given as product concentration and as% (w/w) of product produced from acceptor used. ND; the molar concentration of sialylated GOS and IMO could not be calculated since the distribution of different chain lengths was not determined. Abbreviations: SA, sialic acid; Gal, galactose; Glc, glucose; Fuc, fucose; Fru, fructose.
doi:10.1371/journal.pone.0083902.t002

Table 3. Bacterial growth on sialylated glycans.

Bacterial strain	Area under the growth curve [OD_{600} x min]							
	MRS-	S-GOS	S-fucose	S-melibiose	S-lactulose	S-maltose	S-IMO	galactan
B. infantis 233	30±14	132±5	71±6	14±6	109±20	30±4	95±23	55±7
B. infantis 2238	294±68	274±18	285±15	302±2	269±20	158±24	269±6	264±7
B. infantis 1497	31±10	42±13	149±2	ND	34±2	ND	45±1	40±9
B. longum 232	79±20	162±9	192±20	104±17	134±19	122±31	107±19	42±13
B. lactis	139±70	176±18	192±27	ND	122±18	102±8	175±13	143±15
L. acidophilus	180±28	159±4	188±18	192±4	128±2	193±12	217±19	371±10
B. longum 9917	106±30	71±5	114±15	70±8	34±14	101±6	103±9	93±44
C. perfringens	455±32	722±52	811±48	ND	541±17	844±99	1098±61	447±46

Area under the growth curve for growth of probiotic strains and pathogenic *Clostridium perfringens* on sialylated glycans; MRS- represents growth in the medium with no carbohydrate added; galactan, an established prebiotic, was used as a positive control; growth responses for the substrates are shown for a substrate concentration of 10 g/L for all bacterial strains. Data are given as average values of 3 replicates and shown ± s.d. The growth of *B. longum infantis* 1497, *B. lactis* and *C. perfringens* was not tested on sialylmelibiose, as well as, growth of *B. longum infantis* 1497 on sialylmaltose (ND).

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in modulation of the immune system will need to be addressed in future work.

Concluding Remarks

Previous work by Paris and co-workers [4] identified important residues in the acceptor binding site and in the sialic acid-binding pocket that conferred trans-sialidase activity to the strict sialidase of *T. rangeli*. However, these mutants all retained a much higher (at least 100-fold) hydrolase activity compared to the native trans-sialidase of *T. cruzi*, indicating that a major determinant of impaired hydrolysis in the evolution of trans-sialidase was missing.

In this study, we have shown that a major chemical and structural difference between the native trans-sialidase TcTS and the homologous sialidase TrSA lies in the presence of a +3 charged VTNKKKQ motif on the edge of the acceptor binding cleft, and that this motif effectively reduces hydrolysis to promote trans-sialidase activity. The most likely function of this motif is to effectively disrupt (perhaps even reverse) the water binding network, which must be aligned with the oxygen lone pairs towards sialic acid in order to accomplish hydrolysis. Since the motif is not conserved among characterized trans-sialidases its beneficial effect might be context-dependent. Further structural and enzymatic studies will certainly be needed to assess the validity and generality of the proposed mechanisms.

A mutant, Tr13, was constructed from the parent Tr6 mutant, with this identified motif incorporated and it was shown to display a 4-fold lower hydrolase activity on 3′-sialyllactose, while trans-sialidase activity was essentially unaffected. As a result, a net doubling of the yield of sialylated product was obtained using this particular mutant Tr13, which also produced a better yield than a series of controlling single-site mutants. The mutant is therefore the first in a new class of envisioned mutants capable of reducing hydrolysis in competition with trans-sialidase activity by disruption of the water network of the acceptor-binding site and active site. Tr13 is also remarkable by being chemically substantially different from its wild type, with 13 sites mutated and a charge difference of +3.

Furthermore, the native TcTS is generally believed to use only terminal galactose as acceptor substrates [29], but Tr13 possessed an unusually broad acceptor-substrate specificity, accommodating terminal galactose but also terminal glucose and even monomers of glucose and fucose. This, together with the substantially reduced hydrolysis, should make Tr13 and its downstream mutants useful for enzymatic sialylation of a broad range of glycans in the pursuit of novel, functional food ingredients. To justify this approach, a number of established and potential prebiotics, including GOS, IMO, and lactulose, were sialylated in reasonable yields. An initial examination of prebiotic potential demonstrated growth of Bifidobacteria, although not selectively. Using Tr13 under optimal conditions should further allow a synthesis-scale of these novel molecules enabling further functionality tests including their prebiotic effect in mixed culture, and their potential as anti-adhesive antimicrobial and modulator of the immune system.

Supporting Information

Figure S1 The gene sequence and primary structure of Tr6. Restriction sites used for vector construction are underlined. pPICZαC (Invitrogen)-encoded N-terminus containing α-factor signal sequence and Kex2 and Ste3 protease recognition sequence (amino acids 1–89) and C-terminus containing *c-myc* and 6xHis-tag (amino acids 730–752) in grey. Tr6 gene product (amino acids 90–729) in bold.
(PDF)

Figure S2 Trans-sialidase activity of Tr6 and derived mutants. Trans-sialidase activity measured using cGMP as sialic acid donor and methylumbellferyl-pyrogalactoside as acceptor. Product formation, for each of the mutant variants shown against that of the parent Tr6, is shown in arbitrary units. Differences in initial reaction rate might in part relate to differences in enzyme amount used.
(PDF)

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Author Contributions

Conceived and designed the experiments: CJ MM JDM KPK. Performed the experiments: CJ MM DML HL YG. Analyzed the data: CJ MM ASM JDM. Contributed reagents/materials/analysis tools: JDM ASM FK. Wrote the paper: CJ MM.

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10.3 Expression and characterization of an endo-1,4-ß-galactanase from *Emericella nidulans* in *Pichia pastoris* for enzymatic design of potentially prebiotic oligosaccharides from potato galactans

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Expression and characterization of an endo-1,4-β-galactanase from *Emericella nidulans* in *Pichia pastoris* for enzymatic design of potentially prebiotic oligosaccharides from potato galactans

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ABSTRACT

Potato pulp is a high-volume side-stream from industrial potato starch manufacturing. Enzymatically solubilized β-1,4-galactan-rich potato pulp polysaccharides of molecular weights >100 kDa (SPPP) are highly bifidogenic in human fecal sample fermentations *in vitro*. The objective of the present study was to use potato β-1,4-galactan and the SPPP as substrates for enzymatic production of potentially prebiotic compounds of lower and narrower molecular weight. A novel endo-1,4-β-galactanase from *Emericella nidulans* (anamorph *Aspergillus nidulans*), GH family 53, was produced in a recombinant *Pichia pastoris* strain. The enzyme was purified by Cu^{2+} affinity chromatography and its optimal reaction conditions were determined to pH 5 and 49 °C via a statistical experimental design. The specific activity of the *E. nidulans* enzyme expressed in *P. pastoris* was similar to that of an endo-1,4-β-galactanase from *Aspergillus niger* used as benchmark. The *E. nidulans* enzyme expressed in *P. pastoris* generated a spectrum poly- and oligosaccharides which were fractionated by membrane filtration. The potential growth promoting properties of each fraction were evaluated by growth of beneficial gut microbes and pathogenic bacteria. All the galactan- and SPPP-derived products promoted the growth of probiotic strains of *Bifidobacterium longum* and *Lactobacillus acidophilus* and generally did not support the propagation of *Clostridium perfringens* in single culture fermentations. Notably the growth of *B. longum* was significantly higher ($p < 0.05$) or at least as good on galactan- and SPPP-derived products as fructooligosaccharides (FOS). Except in one case these products did not support the growth of the pathogen *Cl. perfringens* to any significant extent.

1. Introduction

Potato pulp is primarily made up of the cell walls of the potato tuber and is a poorly utilized by-product available in high amounts from industrial potato starch manufacturing. In Europe, potato starch production is tightly regulated by a quota system and potato pulp is mainly used directly as cattle feed and sold to farmers for less than 10 Euros per ton [1]. In order to maintain the economical competitiveness of the potato starch industry improved valorization of the pulp is important. The main polysaccharides of potato pulp are pectin, cellulose, hemicelluloses, and some residual starch [1,2]. The monomeric composition of destarched potato pulp is dominated by galactose, but also includes galacturonic acid, arabinose, and rhamnose indicating that the cell wall material in potato pulp is mainly made up of pectin, primarily rhamnogalacturonan I (RGI) with large galactan rich side chains and homogalacturonan (HG) [2]. HG consists of linear stretches of α-1,4-linked D-galacturonic acid residues which can be methyl esterified at C-6 and/or O-acetylated at O-2 and/or O-3 [3,4]. The degree of methylation and acetylation in potato pectin has been reported to 31 and 14%, respectively [5]. The RGI backbone consists of repeats of the disaccharide [→2)-α-L-rhamnose(*p*)–(1→4)-α-D-galacturonic acid(*p*)-(1→] and may have extensive side chains attached to the O-4 of the rhamnose residues. The side chains are composed principally of arabinose and galactose and consist of α-1,5-linked arabinosyl, β-1,4-linked galactosyl and/or arabinogalactan structures [6]. All three types of side chains are present in potato RGI [7], but β-1,4-linked galactopyranosyl bonds dominate, which is why the side chains of potato RGI are considered to mainly consist of β-1,4-galactan chains, but with some of the galactopyranosyl residues substituted at C-6 by α-1,5-arabinans [8,9].

Enzymatically solubilized polysaccharides from potato pulp have been demonstrated previously to exert potentially beneficial

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properties as dietary fibers and prebiotics in both *in vitro* (human fecal fermentations) and *in vivo* (rat and human) studies [10–12]. Recently, the enzyme catalyzed polysaccharide solubilization process from potato pulp has been optimized further resulting in release of high yields of pectinaceous polysaccharides with high molecular weights, up to 400 kDa, via a one-step enzymatic process using pectin lyase and polygalacturonase from *Emericella nidulans* (formerly known as *Aspergillus nidulans*) and *Aspergillus aculeatus*, respectively [12,13]. These soluble, high molecular weight potato pulp polysaccharides (SPPP), which are presumably made up of mainly RGI β-1,4-galactan chains, appeared to be particularly promising as prebiotics as this substrate selectively and significantly increased the densities of *Bifidobacterium* spp. and *Lactobacillus* spp. when fermented *in vitro* by microbial communities in human fecal samples; the stimulation of the *Bifidobacterium* spp. was moreover better than that induced by commercially available fructo-oligosaccharides (FOS, DP 2–8 Orafti®P95) used as a positive control [12].

Different types of galacto-oligosaccharides are already well recognized in the literature for their bifidogenic and prebiotic effects. We hypothesized that further enzymatic digestion of the SPPP to form β-1,4-galactan derived poly- and oligo-saccharides of lower, and more narrow, molecular weights might unravel the ultimate health benefits of β-1,4-galactans from potato pulp. In order to targetly produce such galactosaccharides we produced and explored a new cloned endo-1,4-β-galactanase from *E. nidulans* and examined the optimal reaction conditions and product profiles generated. Our aim of the present study was therefore to use the high molecular weight potato pulp polysaccharides, SPPP, as a substrate for further targeted enzyme catalysis to provide new opportunities for designing functional food ingredients with significant health benefits and at the same time provide for a valorization route for potato pulp.

2. Materials and methods

2.1. Substrates and enzymes

Galactan from potato was purchased from Megazyme International LTD (Bray, Co. Wicklow, Ireland). Potato pulp was supplied by Lyckeby Stärkelsen (Kristianstad, Sweden). The high molecular weight solubilized potato pulp polysaccharides (SPPP) were prepared from crude potato pulp via a short, minimal enzyme treatment with pectin lyase and polygalacturonase, as described previously [12,13] (the SPPP were coded as CPP > 100 in [12]).

The endo-1,4-β-galactanase (EC 3.2.1.89) from *E. nidulans* (GB accession no. ABF50874.1; Swiss Prot Q5B153) was produced by fermentation in *Pichia pastoris* as outlined below. The clone was supplemented by FGSC, and had been constructed from the chromosomal DNA of *A. nidulans* FGSC A4 (*E. nidulans*) as described by Bauer et al. [14]. Endo-1,4-β-galactanase from *A. niger* was purchased from Megazyme Intl LTD.

2.2. Fermentation of endo-1,4-β-galactanase from E. nidulans in P. pastoris

A 5 L Sartorius Biostat Aplus fermentor was inoculated with *P. pastoris* cells grown for 20 h at 30 °C, at 150 rpm in shaking flasks with minimal glycerol medium as detailed by Stratton et al. [15]. The 5 L scale production of recombinant protein in *P. pastoris* was done essentially as described in [15] and as detailed by Silva et al. [16], except that the temperature for the Glycerol Batch and Glycerol Fed-Batch phases was 30 °C and then adjusted to 25 °C for the Methanol Fed-Batch phase in order to improve the stability of the enzyme. Agitation was specifically controlled below 750 rpm to avoid excessive cell debris from the *P. pastoris* cells and to limit the downstream purification process. Additional oxygen was added automatically to accommodate optimal growth and enzyme expression. The total time of fermentation was 95 h. The biomass (OD_{600}) and protein induction (by the CA assay explained below) was followed throughout the fermentation process by off-line sampling as explained in [16].

2.3. Downstream process

At the end of the fermentation process the *Pichia* cells were collected by centrifugation at 5300 × *g*, 5 °C for 1 h. The supernatant was then subjected to sterile filtration, followed by concentration by ultrafiltration using a cross-flow bioreactor system with a 10 kDa cutoff membrane (Millipore, Sartorius) as described in [16]. The enzyme preparations containing 25% (w/v) glycerol were stored at −80 °C.

2.4. Gel electrophoresis and Western blotting

SDS-PAGE was carried out using a Mini-Protean® Tetra System, Bio-Rad Laboratories (California, USA). The gel was a Mini-Protean® TGX™ 12%. The running buffer was Tris/glycine/SDS. The power conditions were: 150 V, run time: 60 min, starting current: 20 mA and final current: 3A. The proteins were visualized by staining with Comassie brilliant blue.

Selected proteins were digested with endoglycosidase H (EndoH, Medinova) for 60 min at 37 °C before they were electrophoretically transferred to PVDF (Hybond-LFP) membranes using the standard wet transfer method (10% methanol, 14.4 g/L glycine and 3 g/L Tris buffer). After blocking with defatted milk proteins, the membranes were incubated with monoclonal anti-poly-histidine-peroxidase antibodies (Sigma–Aldrich), TBS, milk and Tween20 solution. Chemiluminescence was induced with Amersham ECL Plus Western Blotting Detection Reagents and the signals were captured by exposure to Hyperfilm-ECL (Amersham).

2.5. Determination of protein concentration

The protein concentration was determined via reaction with bicinchoninic acid (BCA) using The Thermo Scientific (Rockford, USA) Pierce® BCA Protein Assay Kit according to the manufacturer's instructions. Bovine serum albumin was used as a standard.

2.6. Enzyme purification

The purification was performed by Cu^{2+} affinity column chromatography using a CIM® IDA-8f ml Tube Monolithic Column purchased from BIA Separations GmbH (Villach, Austria). The affinity chromatography was carried out as described previously [16]. The imidazole was removed using PD-10 columns (GE Healthcare).

2.7. Reducing sugars assay

The enzymatic hydrolysis of galactan was assessed by a reducing sugars assay modified from Lever [17,18]. The mixture of enzyme and substrate was incubated at 50 °C, pH 5 for 30 min. The substrate concentration was 6 g/L and the enzyme/substrate ratio (E/S) (v/w) was 0.3%. The reaction was stopped by heating for 10 min at 100 °C. 20 μl of filtered sample was added to 3 ml of an alkaline *p*-hydroxybenzoic acid hydrazide (PAHBAH) solution. Under alkaline conditions PAHBAH reacted with reducing ends of saccharides giving yellow anions. The concentration of reducing ends was calculated from the absorbance at 410 nm using D-(+)-galactose (Sigma–Aldrich, Steinheim, Germany) as the standard. One unit of enzyme activity was defined as production of 1 μmol/ml min of reducing ends (galactose equivalents) in the assay mixture; and 1 kU = 1 M (gal equiv)/min.

2.8. Statistical design of the optimal conditions for hydrolysis of galactan from potato by endo-1,4-β-galactanase from E. nidulans expressed in P. pastoris

The endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was incubated with a solution containing galactan from potato in 5 mM NaH_2PO_4 (Sigma–Aldrich, Steinheim, Germany). Three different pH regimes 4, 5 and 6 were tested. Incubation temperatures were 40, 50 and 60 °C. The time of incubation was 1, 4.5 and 8 h. The tested enzyme to substrate ratios E/S (v/w) were 0.1, 0.3 and 0.5%. A quadratic central composite design was used. MODDE Version 7.0.0.1 (Umetrics AB, Umeå, Sweden) was used as an aid to design the experimental frame and to fit and analyze the data by multiple linear regression analysis. Before the reaction, the substrate was preincubated for 5 min at the appropriate temperature. The reaction was stopped by heating for 10 min at 100 °C. The concentration of reducing ends was then determined as described above.

2.9. Thermostability of endo-1,4-β-galactanase from E. nidulans expressed in P. pastoris

The thermal stability of the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was evaluated by preincubation of the enzyme in 5 mM NaH_2PO_4 buffer (pH 5) at 50, 60 and 70 °C for 2–60 min. After a set exposure time at the selected temperature, an aliquot of the enzyme solution was cooled to 50 °C, 500 μl of galactan solution was added to achieve an E/S (v/w) of 0.3%, and the residual enzyme activity was measured via the reducing sugars assay as described above.

2.10. Influence of metal ions on enzyme activity

The *E. nidulans* endo-1,4-β-galactanase expressed in *P. pastoris* was incubated with 10 mM EDTA for 40 min at room temperature. The control sample was incubated with 5 mM phosphate buffer pH 5 instead of the EDTA solution. After incubation with EDTA, the EDTA was removed by gel filtration (PD-10 columns, GE Healthcare). The relevant metal ion solution was added (1 mM) to aliquots of EDTA pretreated and control samples, respectively (no metal addition was a benchmark both in the EDTA pretreated and in the control samples), and the reducing sugars assay was used to determine the residual enzyme activity.

2.11. K_M and V_{max} determination of endo-1,4-β-galactanase from E. nidulans expressed in P. pastoris

The apparent K_M and V_{max} values for the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* were determined using galactan from potato. The substrate was incubated in phosphate buffer at pH 5 for 5 min at 50 °C. The enzyme was added and samples were taken every minute for 5 min. The substrate concentrations were varied to result in different E/S (v/w) levels of 0.3–1.7%. The activity of the enzyme was determined by the reducing ends assay described above.

2.12. Time-course hydrolysis of galactan polymers

The endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was incubated at its optimal temperature and pH with galactan (E/S (v/w) = 0.3%) for 15, 30, 60, 120 and 270 min in 5 mM NaH_2PO_4. The polymer degradation over time was evaluated by HPSEC analysis as detailed below.

2.13. 100 ml-scale enzymatic hydrolysis of potato galactan and SPPP

The reactions were performed in 100-ml scale to obtain sufficient amounts of oligosaccharides to perform *in vitro* growth curve determination. The enzymatic reaction was performed at the conditions optimal for the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris*. Four 50 ml tubes each containing 25 ml of substrate solution in buffer were preincubated for 5 min at 50 °C. The reaction was carried out for 15, 60 or 270 min at an E/S ratio of either 0.3% or 0.03%.

2.14. Membrane separation of the products from the enzymatic hydrolysis

After the enzymatic hydrolysis the reaction mixtures of oligomers and polymers were separated using polyethersulfone or regenerated cellulose membranes (Millipore, Billerica, MA). The cut-offs of the membranes were 3, 10, 30 and 100 kDa. The filtration was carried out in a stirred membrane reactor with a constant pressure at 4 bar (Millipore, Billerica, MA). The reactor content was stirred carefully to decrease fouling. From each reaction mixture two fractions were obtained. In case of galactan, each mixture was filtered two times, with 2 membranes of different cut off; e.g. to create samples PG3 and PG4 a membrane of 100 kDa was applied to narrow the size of the investigated compounds and to remove the parts of the substrate, which were not hydrolyzed by the applied enzyme. Subsequently a membrane of 10 kDa cut-off was used. When SPPP was used as a substrate for the reaction mixture, the separation was performed using only 1 membrane of cut-off 10 kDa, since the products of the reaction of molecular weight higher than 100 kDa were the most abundant component of the post-reaction mixture and moreover the high molecular weight compounds of SPPP revealed high bifidogenic properties in fermentation *in vitro* in human fecal samples [12]. The yield of the obtained fractions was calculated in percentage as weight of produced sample to weight of starting material. The molecular weights of the poly- and oligomers in the permeates and retentates were evaluated by HPSEC analysis as described below.

2.15. Analytical methods

High-performance size exclusion chromatography (HPSEC): HPSEC was carried out as described in [19] using a Shodex SB-806 HQGPC column (300 mm × 8 mm) and a Shodex SB-G guard column (50 mm × 6 mm) from Showa Denko K.K. (Tokyo, Japan) on a system consisting of a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex Corp., Sunnyvale, CA), but with the modification that the mobile phase was a 0.5 M sodium acetate solution, pH 6. The molecular mass markers were dextrans of molecular masses 110×10^3 g/mol, 70×10^3 g/mol, 40×10^3 g/mol, 17.2×10^3 g/mol, 10×10^3 g/mol and pullulan of molecular mass 6×10^3 g/mol.

High-performance anion exchange chromatography (HPAEC-PAD): Separation and quantification of oligosaccharides were carried out by HPAEC-PAD analysis using a Dionex BioLC system (Dionex Corp., Sunnyvale, CA) principally as described in [20], but with a slightly changed elution program of the three eluent system comprised of deionised water (A), 0.5 M NaOH (B), 0.5 M NaOAc (C). For the first 5 min a linear gradient from 80:20 (%A:B) to 70:20:10 (%A:B:C) was applied, which was followed by an isocratic elution with 70:20:10 from 5 to 30 min. From 30 to 35 min a linear gradient was applied from 70:20:10 to 70:20:20 and then isocratic elution from 35 to 45 min with 70:20:20. Then, isocratic elution with 20:80 (%B:C) was applied for 10 min to remove strongly retained anions and the column was re-equilibrated for 10 min with 80:20 (%A:B).

The monosaccharide compositions of the galactan and SPPP fractions were determined by a mild, extended acid hydrolysis method modified from Garna et al. [21]: At a concentration of 2.5 g/L each substrate was treated with 0.2 M trifluoric acid for 72 h at 80 °C. The recovery of monosaccharides was determined by performing the same hydrolysis on L-rhamnose, L-arabinose, D-galactose, D-glucose, and D-galacturonic acid. The samples were then filtered through a 0.2 μm filter prior to HPAEC-PAD analysis (described below) and the recovery factors obtained were used to quantify the monosaccharides.

The separation and quantification of monosaccharides were done by HPAEC-PAD analysis using an ICS-3000 system (Dionex Corp., Sunnyvale, CA) equipped with a CarboPac™ PA20 (3 mm × 150 mm) column and a CarboPac PA20 (3 mm × 30 mm) guard column as described in [2], but by use of a slightly modified elution profile: After injection of the sample a linear gradient from 15 to 5 mM NaOH was applied from 0 to 1.5 min, followed by isocratic elution with 5 mM NaOH from 1.5 to 3 min. From 3 to 7 min a linear gradient from 5 to 2.5 mM NaOH was used and from 7 to 17 min another isocratic elution was applied with 2.5 mM NaOH followed by a third isocratic elution with 500 mM NaOH from 17 to 35 min. External monosaccharides standards were D-galactose, D-glucose, L-rhamnose, L-arabinose, D-galacturonic acid, and D-mannose.

Thin layer chromatography (TLC) analysis: The conversion of galactan to oligosaccharides was followed by TLC using silica gel 60 plates from Merck (Darmstadt, Germany). The solvent system was 1-butanol:formic acid = 2:3. The products were visualized by spraying the plate with 10% (v/v) sulfuric acid in ethanol and subsequently charring it at 130 °C.

2.16. Bacterial growth assays on the prebiotic candidates and reference compounds

The substrate samples were dissolved in water at 10% (w/v) and sterilized by UV-radiation for 30 s. The bacterial strains were *Lactobacillus acidophilus* (NCFM, ATCC 700396); *Bifidobacterium longum* (Bl-05, Danisco Global Culture Collection, DGCC 9917); *Bifidobacterium lactis* (HN019, DGCC2013); *Escherichia coli* (ATCC 11775); and *Clostridium perfringens* (ATCC 13124). These strains were incubated in mono-cultures with the individual substrates at concentrations of 1% (w/v) in designated multiwell plates in a Bioscreen® C system (Labsystems, Helsinki, Finland) as described previously [22]. Anaerobic growth was measured by monitoring the increase of the biomass by optical density measurement at 600 nm (OD_{600}) using Biolink® software (Labsystems) according to the protocol detailed in [22]. The bacterial growth was determined as a function of OD_{600} and time. The baseline growth in the media without addition of carbohydrates was used as control and subtracted from growth obtained in the presence of substrate [22,23]. Glucose and galactose were used as non-selective control substrates. Fructooligosaccharides (Raftilose®, Beneo, Belgium) were used as an established prebiotic standard control. Two sets of experiments were done in three replicates for each strain and carbohydrate substrate. Data are given as mean values ± standard error.

2.17. Statistics

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

3. Results and discussion

3.1. Fermentation of endo-1,4-β-galactanase from E. nidulans in P. pastoris

Throughout the high cell density fermentation of *P. pastoris* containing the recombinant endo-1,4-β-galactanase the biomass of *P. pastoris* was followed by measurement of the OD_{600}, which increased from 2 to 630 during the 95 h fermentation process (Fig. 1). The level of extra-cellular protein including the endo-1,4-β-galactanase reached 2.11 g/L after 95 h (Fig. 1). The protein expression was followed by SDS-PAGE and the endo-1,4-β-galactanase protein band at 39 kDa was observed shortly after methanol induction of the AOX1 promoter controlling the expression of the gene. The SDS-PAGE also showed that appreciable amounts of endogenous protein from *P. pastoris* were present in the extract. The endo-1,4-β-galactanase activity at the end of the fermentation was 109 kU/L (equivalent to at total of 393 kU in the 3.6 L fermentation volume).

3.2. Enzyme purification

The endo-1,4-β-galactanase produced in *P. pastoris* was not pure, but contained several other protein bands (Fig. 2). The fermentation was optimized to generate a very low level of cell wall debris and to limit the accumulation of intracellular proteins from *P. pastoris* in the supernatant. This improved the down-stream processes, sterile-filtration and ultra-filtration, but was not sufficient to eliminate all the protein impurities. A final purification of the enzyme was therefore necessary. The endo-1,4-β-galactanase was purified on a CIM-Cu^{2+} affinity column using the His-Tag

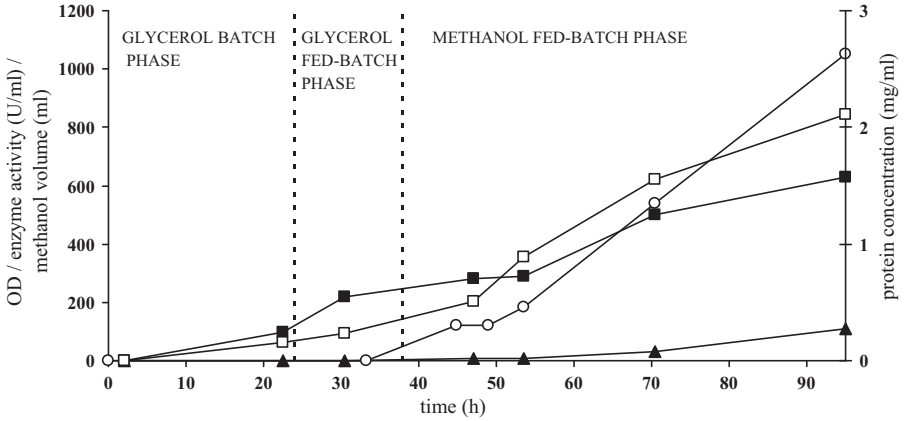

Fig. 1. Evolution of key parameters during the 95 h fermentation of *P. pastoris* to produce the recombinant endo-1,4-β-galactanase from *E. nidulans*: Biomass measured as optical density at 600 nm (OD_{600}) (■); enzyme activity (▲), and the methanol added during the fermentation (○) shown on the left *Y*-axis; protein concentration (□) shown on the *Y*-axis to the right.

binding module and an Äkta system [16]. The bound proteins were desorbed with imidazole buffers and the combined fractions were analyzed by SDS-PAGE and Western blot analysis. The yield of the purified enzyme after the affinity column chromatography was 82%, and the overall yield of endo-1,4-β-galactanase was 1.6 g/L. This level of heterologous expression is high compared with the levels for approx. 200 proteins listed by Cereghino and Cregg [24], where only 7% have a titer higher than 1 g/L.

The endo-1,4-β-galactanase belongs to GH family 53 as categorized in Carbohydrate-Active enZymes (CAZy) Database [25] (the enzyme works via a retaining mechanism and the structure of the GH53 domain is known to have a TIM barrel fold). The molecular mass of the endo-1,4-β-galactanase as determined from the amino acid sequence (Expasy Parameter program) was 38.6 kDa, in accordance with the finding that the band obtained after purification was 39 kDa (Fig. 2). A glycosylation was therefore not expected, although it is common among enzymes produced in *P. pastoris*. Further analysis by Western blot and treatment with EndoH confirmed that the MW did not decrease which also indicated that the endo-1,4-β-galactanase was not glycosylated (Fig. 2).

Alignment of the amino acid sequence from endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* with sequences from other endo-1,4-β-galactanases using the blast program in the NCBI server (http://www.ncbi.nlm.nih.gov) gave the highest identity of 71–77% with enzymes from *Aspergillus tubingensis*, *A. niger*, *A. terreus*, and *Penicillium chrysogenum* (data not shown). Prediction of the glycosylation sites by NetNGlyc tools (expasy.org/tools) indicated two potential glycosylation sites in the endo-1,4-β-galactanase, whereas a glycosylated rhamnogalacturonan I lyase from *Bacillus licheniformis* expressed in *P. pastoris* was recently found to have 4 potential sites [16].

3.3. Optimal temperature and pH

To determine the pH and temperature optimum of the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* a statistically designed, quadratic central composite experiment was applied to evaluate the individual and interactive effects of 4 factors (pH, temperature, enzyme to substrate ratio, E/S (v/w), and reaction time) in 24 experimental combinations. The optimal pH and temperature combination was determined to be pH 5 and 49 °C the regression model forming an almost symmetric bell-shaped response surface optimum (Fig. 3). The optimal pH of 5 was in agreement with the findings of Bauer et al. [14], but the relatively high optimal temperature has not been determined previously. As expected, increasing the ratio of E/S (v/w) increased the yield of reducing ends. The same was observed with increased reaction time up to 8 h, despite that the enzyme lost half of the activity in 27 min at 49 °C (Fig. 4); the half life, $t_{½}$ of the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was 57 min at 40 °C, 27 min at 49 °C and ~10 min at 60 °C as assessed in phosphate buffer (Fig. 4). Since the temperature optimum of the investigated enzyme was 49 °C (in the presence of substrate) and a relatively low thermal stability was observed for the enzyme when incubated without the substrate at elevated temperatures, the results strongly indicate that the endo-1,4-β-galactanase is very stable in the presence of the substrate.

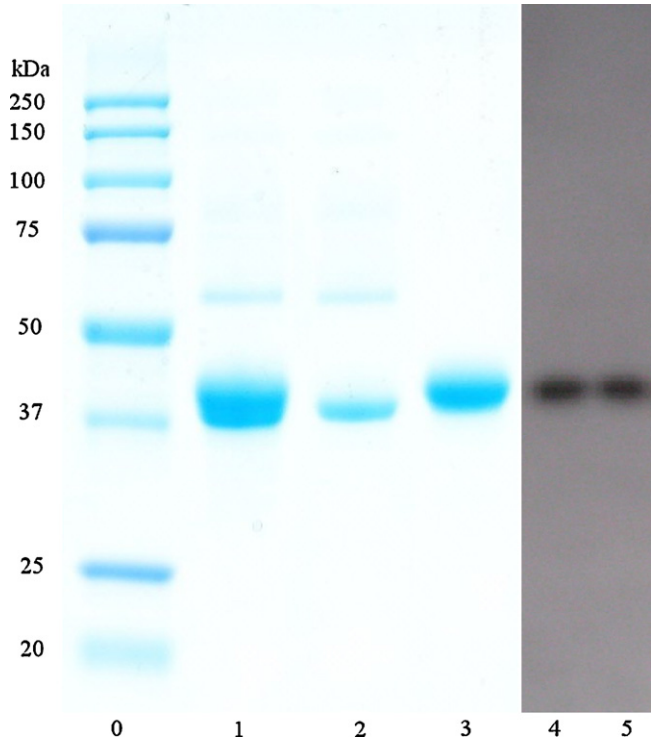

Fig. 2. SDS-PAGE of enzyme samples from the separation steps; lane 0, molecular mass marker; lane 1, crude enzyme preparation; lane 2, run off fraction from the Cu^{2+} affinity column; lane 3, pure enzyme eluted by the imidazoline buffer; lane 4, Western blot of pure enzyme; and lane 5, Western blot of pure denatured enzyme after treatment with EndoH.

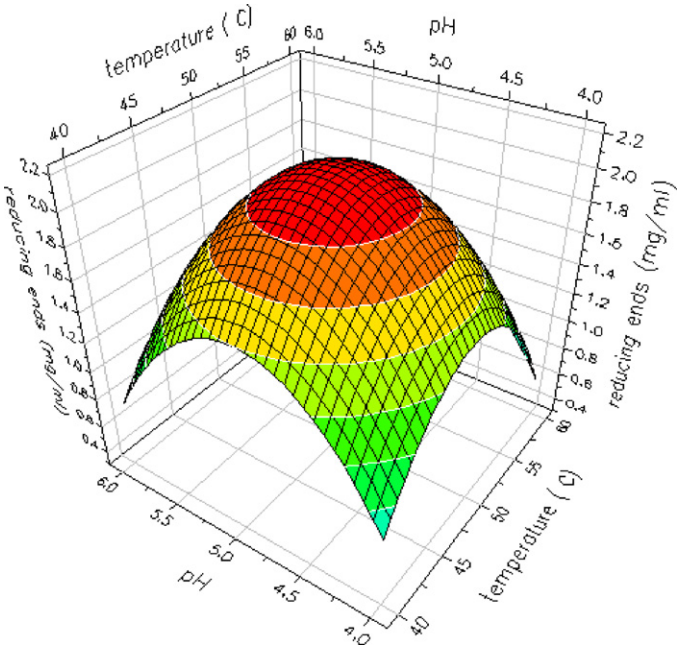

Fig. 3. Surface response as a function of temperature and pH of the endo-1,4-β-galactanase activity. The colors vary from turquoise/green (low endo-1,4-β-galactanase activity) to red (high endo-1,4-β-galactanase activity). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Kinetic parameters and metal ions interactions

The kinetic parameters K_M and V_{max} for endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* were determined to 0.51 g/L and 73.3 U/mg protein, respectively using a Hanes plot (data not shown).

The catalysis of endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* was compared with that from *A. niger*. The optimal pH for the latter enzyme was 4–4.5, and the optimal temperature was 40 °C. The specific activity of the endo-1,4-β-galactanase from *A. niger* was 70 U/mg (concentration 4.3 mg/ml in the purchased preparation). Hence, the specific activity of the endo-1,4-β-galactanase *E. nidulans* expressed in *P. pastoris* and the specific activity of enzyme from *A. niger* were similar. The influence of metal ions on the *E. nidulans* enzyme activity was also tested. The enzyme was pretreated with EDTA prior to incubation with the specific metal ions (and the EDTA pre-incubation was compared to the results obtained without EDTA added). Except for the significantly lower activity in the presence of $AlCl_3$, the endo-1,4-β-galactanase activity was not affected by the metal ions (Table 1). The influence of metal ions on fungal endo-β-1,4-galactanases performance described in the literature is usually observed as weak or inhibitory. Pb^{2+} and to a lesser extent Zn^{2+} and Ag^{+} have previously been found to decrease the activity of endo-β-1,4-galactanases from *A. niger* and *A. aculeatus* [9], and the activity of an endo-β-1,4-galactanase from *A. sojae* has also been reported to be significantly inhibited by Mn^{2+}, Hg^{2+}, Ag^{+}, and Fe^{3+} [26]. The fact that the majority of metal ions had no effect on the activity of endo-1,4-β-galactanase from *E. nidulans* produced in *P. pastoris* could make this enzyme better suited for the industrial applications than the other endo-galactanases discussed above.

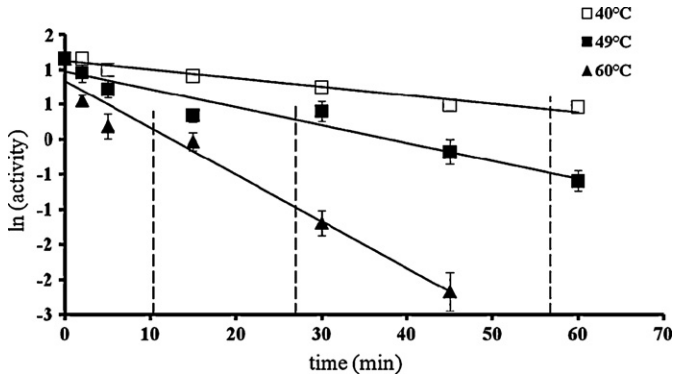

Fig. 4. Thermal stability of endo-1,4-β-galactanase from *E. nidulans*; dashed lines represent enzyme half lives, $t_{½}$, for 60 °C (10 min), $t_{½}$ for 49 °C (28 min) and $t_{½}$ for 40 °C (57 min). Thermal stability was assessed in phosphate buffer.

Table 1
Influence of metal ions on enzyme activity. Different superscript letters a–c indicate significantly different values at $p < 0.05$ (one way ANOVA, pooled S.D. 3.7).

Pretreatment	Metal added	Activity (%)
None	None	100 ± 3.6^{a}
None	$AlCl_3$	73.3 ± 6.4^{b}
None	$MgCl_2$	92.7 ± 3.3^{a}
None	$MnCl_2$	89.2 ± 1.9^{a}
None	$ZnCl_2$	105.7 ± 4.9^{a}
None	$CaCl_2$	98.7 ± 5.2^{a}
EDTA	None	87.7 ± 1.2^{a}
EDTA	$AlCl_3$	51.3 ± 3.3^{c}
EDTA	$MgCl_2$	90.8 ± 4.9^{a}
EDTA	$MnCl_2$	104.4 ± 0.3^{a}
EDTA	$ZnCl_2$	107.5 ± 2.2^{a}
EDTA	$CaCl_2$	103.5 ± 2.7^{a}

3.5. Release of poly- and oligosaccharides from potato galactan

The optimized conditions established above for endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* were used to create potentially prebiotic poly- and oligosaccharides by enabling enzyme catalyzed hydrolysis of the potato galactan polymer and the SPPP substrate, respectively. The galactan polymer had a molecular mass of about 150 kDa, and the composition of monosaccharides (in mol%) was 86% galactose, 4% rhamnose, 7% arabinose, 3% galacturonic acid. The galactan polymer was progressively enzymatically degraded to lower molecular weight products in time course experiments (2–270 min) using E/S ratios of 0.03% and 0.3% (Fig. 5). The molecular weights (MW) of the products were examined by HPSEC, and the experimental conditions yielding the optimal level of the products were selected for further studies at 100-ml scale reactions. Galactan derived poly- and oligosaccharides were separated according to molecular weight using 3 kDa, 10 kDa or 100 kDa membranes. A 3 kDa permeate referred to as PG1 (potato galactan, 60 min; E/S 0.03%) was obtained with a yield of 28.7% (172 mg) and contained oligosaccharides made up of 95% galactose and 5% arabinose (mol%). PG2 was the retentate corresponding to PG1; the hydrolyzate had been filtered through a 10 kDa membrane prior to the 3 kDa membrane filtration, and PG2 thus contained saccharides of 3–10 kDa (Fig. 5b); the molar composition of PG2 (yield 30%) was 93% galactose, 6% arabinose, 1% rhamnose.

PG3 was a 10 kDa permeate produced by 15 min hydrolysis using an E/S ratio of 0.3%. The yield was 67% (402 mg) and the sugar composition (mol%) was 93% galactose, 6% arabinose and 1% rhamnose. PG4 was the retentate of PG3 (Fig. 5), and contained oligo- and polysaccharides of ~10–100 kDa (obtained at a yield of 29% by weight) with a molar composition of 88% galactose, 8% arabinose, 2.5% rhamnose, and ~1.5% galacturonic acid.

PG5 was the 3 kDa permeate produced by an extended, intensive endo-β-1,4-galactanase treatment of galactan for 270 min at an E/S ratio of 0.3%. The yield was 43% (257 mg) and the

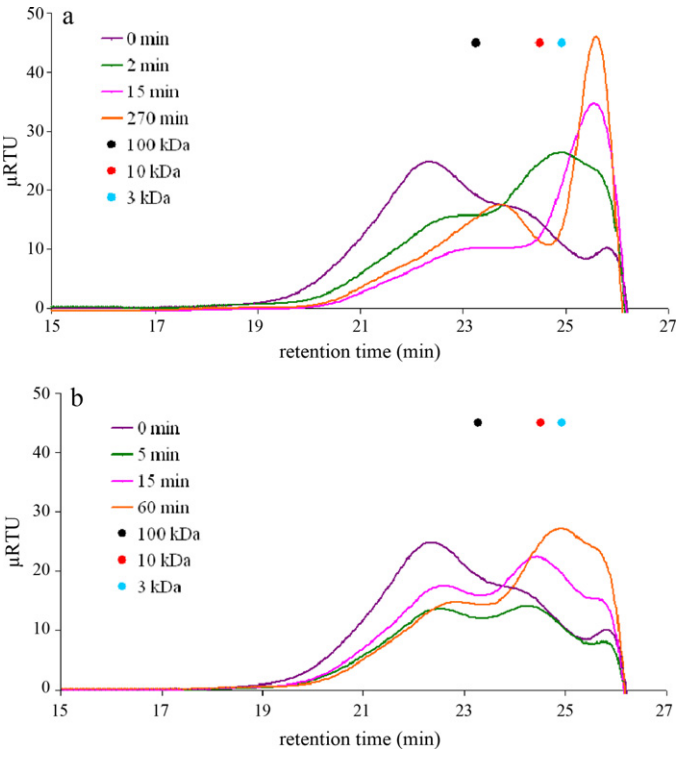

Fig. 5. HPSEC analysis after enzymatic hydrolysis of potato galactan (Megazyme): (a) E/S (v/w) = 0.3% and (b) E/S (v/w) = 0.03%.

composition was ~93% galactose, 7% arabinose and 0.3% rhamnose. The yields and composition of all samples are summarized in Table 2. The content of free galactose was relatively high in both of the 3 kDa fractions, i.e. in PG1 and PG5. The high amounts of low molecular weight galactose oligosaccharides were further analyzed by HPAEC, where accumulation of galactose and galactobiose coincided with extended incubation time and high E/S ratio of 0.3% E/S (Fig. 6). One possibility for the high galactose released could be that the endo-β-1,4-galactanase from *E. nidulans* expressed in *P. pastoris* acted according to a multiple attack mechanism and that the monosaccharide release therefore was an "accidental" result of multiple attacks on the substrate as detailed by van de Vis [9]. Another explanation may be that the enzyme had an ability to exhibit exo-β-1,4-galactosidase activity (EC 3.2.1.23) as the reaction progressed. Similar findings of high galactose levels have been reported in a number of studies with other endo-β-1,4-galactanases derived from *Aspergillus* spp. [9,26–30]. Using the *p*-nitrophenyl-β-D-galactopyranoside as the substrate did not prove the presence of any exo-activity of the enzyme, however (data not shown).

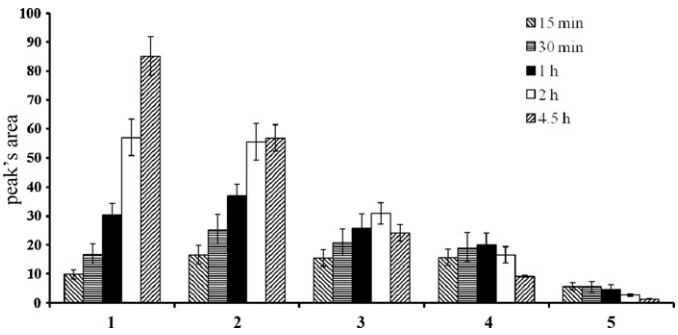

Fig. 6. Evolution profile of oligosaccharides released from potato galactan (Megazyme) by the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris*, used at E/S (v/w) 0.3%, with time: 1, galactose monomer eluted at 4.5 min; 2, galactose dimer eluted at 7.4 min; 3, galactose trimer eluted at 9.6 min; 4, galactose tetramer eluted at 14.5 min; and 5, galactose pentamer eluted at 24.1 min.

Although the potato galactan polymer contained some galacturonic acid (3.5%), the PG fractions obtained were almost devoid of galacturonic acid although a little bit was analyzed in PG4. It is expected that the galacturonic acid and RGI backbone stretches remained intact in the undigested residues of the polymeric substrate. A relatively high galacturonic acid content in some domains of the polymer may render it resistant to enzyme hydrolysis. This phenomenon has been described previously for enzymatic degradation of related polysaccharides [27].

3.6. Release of poly- and oligosaccharides from high molecular weight solubilized potato pulp polysaccharides (SPPP)

The high MW SPPP were prepared from crude potato pulp via a short, minimal enzyme treatment with pectin lyase and polygalacturonase, as described by Thomassen et al. [12,13]. The molecular mass of the SPPP was higher than 400 kDa and the molar monosaccharide composition (mol%) was 66% galactose, 2% rhamnose, 9% arabinose, 14% galacturonic acid, 7% glucose, and 2% mannose. Different samples of oligosaccharides derived from the SPPP were obtained by treatment of this substrate with an E/S ratio of 0.03% in combination with incubation for 60 min or by using an E/S 0.3% and 15 min of incubation. The reaction was conducted in 100-ml scale. The HPSEC chromatograms of the reaction products are detailed in Fig. 7. After incubation for 15 min the reaction mixture was separated by a 10 kDa membrane and two fractions were obtained, the permeate fraction SPPP1 and the 10 kDa retentate (fraction SPPP2). The yield of SPPP1 was 21% by weight (124 mg), and the products had a molar composition of ~93% galactose, 4% arabinose, 3% galacturonic acid, and 0.3% glucose. The majority of the galacturonic acid was thus present in the SPPP2 fraction which constituted 65% by weight (390 mg) of the originally enzyme treated material and had

Table 2
Yields and details of produced galactan and SPPP fractions[a].

Sample	Fraction details	Yield (w/w %)	Composition (mol%)					
			Galactose	Arabinose	Rhamnose	Galacturonic acid	Glucose	Mannose
PG1	Galactan, 0.03%, 60 min, less than 3 kDa	28.7	95.2	4.8	0	0	0	0
PG2	Galactan, 0.03%, 60 min, between 3 and 10 kDa	30.2	92.7	6.4	0.9	0	0	0
PG3	Galactan, 0.3%, 15 min, less than 10 kDa	67.1	93.0	5.6	1.4	0	0	0
PG4	Galactan, 0.3%, 15 min, between 10 and 100 kDa	29.0	87.7	8.4	2.5	1.4	0	0
PG5	Galactan, 0.3%, 270 min, less than 3 kDa	42.9	93.1	6.6	0.3	0	0	0
SPPP1	SPPP, 0.3%, 15 min, less than 10 kDa	20.6	92.9	3.6	0	3.2	0.3	0
SPPP2	SPPP, 0.3%, 15 min, more than 10 kDa	65.0	53.9	20.2	3.1	10.8	7.4	4.6

[a] Samples PG1 and PG2 are from one hydrolysis. The sum of their yields does not give 100.0%, since PG1 is the fraction below 3 kDa and PG2 is the fraction between 3 and 10 kDa and the material above 10 kDa was separated as well. Similarly for samples PG3 and PG4, but in this case only material above 100 kDa was discarded. The galacturonic acid is presumed to be in the un-reacted material that did not pass the membrane during filtration. In case of SPPP, the endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* catalyzed the cleavage in the galactan part of the substrate, therefore galacturonic acid was mainly retained in the SPPP2.

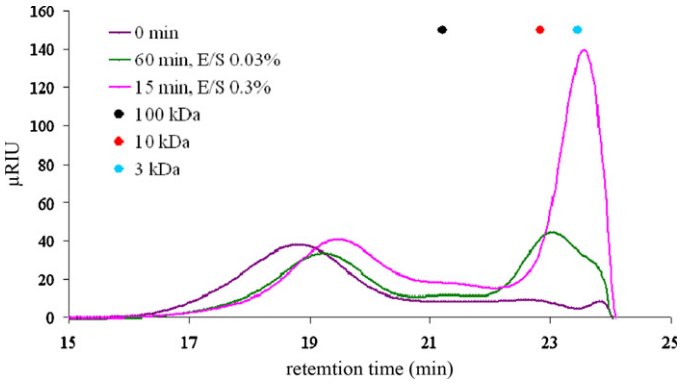

Fig. 7. HPSEC analysis of *E. nidulans* endo-1,4-β-galactanase catalyzed hydrolysis of SPPP.

a monomer composition (in mol%) of 54% galactose, 20% arabinose, 3% rhamnose, 11% galacturonic acid, in addition to 7% glucose and 5% mannose (Table 2).

3.7. Effects on growth in selected mono-cultures

The potential prebiotic properties of the fractionated oligosaccharides generated by enzymatic catalysis of galactan and SPPP, respectively, were evaluated by measuring their growth promoting activity on selected pure cultures using a Bioscreen microtiter system. The growth performance expressed as area under the growth curve [22] was assessed for five different bacteria comprising three probiotic strains, *L. acidophilus*, *B. longum*, and *B. lactis*, one pathogenic strain of *Cl. perfringens*, and one commensal *E. coli* strain. Fructooligosaccharides were used as a control due to their prebiotic properties on bifidobacteria and lactobacilli and their presumed ability to decrease growth of clostridia [31].

The growth of each individual test bacterium was affected differently by the different compounds. In addition, the different bacteria also responded differently to the same substrate (Fig. 8).

The PG5 fraction (PG5 is hydrolyzed galactan <3 kDa, 270 min, E/S 0.3%) stimulated the growth of *L. acidophilus* and *B. longum* significantly better than any of the other hydrolysates; the stimulation of the *L. acidophilus* growth was at the same level as that achieved with FOS but significantly better ($p < 0.05$) than that obtained with pure galactose and unhydrolyzed galactan. The stimulation of *B. longum* was more than 6-fold better than that of FOS and significantly better ($p < 0.05$) than that achieved with galactose or the unhydrolyzed galactan (Fig. 8). The PG1 fraction (<3 kDa, 60 min, E/S 0.03%) and the SPPP1 substrate (<10 kDa fraction generated by digestion of SPPP for 15 min, E/S 0.3%) also stimulated the growth of both *L. acidophilus* and *B. longum* to a significant extent – for *B. longum* the growth stimulation by PG1 and SPPP1 as well as by PG4 was significantly better than that obtained with either FOS, galactose or the original galactan ($p < 0.05$) (Fig. 8). The samples PG1, PG3, PG5, and SPPP1 all contained low molecular weight oligomers and some galactose (data not shown), which might explain some of the growth stimulation of *L. acidophilus* and *B. longum* promoted by these substrates. It is important to note, however, that the growth of *B. longum* was better on all these fractions (PG1, PG4, PG5, SPPP1) than on neat galactose (Fig. 8); this result might indicate that each of these hydrolysates contained particular galactan or arabino-galactan structures, of relatively low molecular weight, which were particularly effective to stimulate the growth of *B. longum*. These structures could be either small linear β-1,4 galacto-oligosaccharides or certain low molecular weight branched structures that were liberated from the galactan (and SPPP) as the enzymatic hydrolysis of the β-1,4-galactan progressed. The high molecular weight fraction SPPP2 was tested only at 0.5% (w/v) due to its low solubility and high viscosity in the solution at this dosage level. It did not support the growth of any of the investigated microorganisms to a high extent (data not shown). The data also showed that the growth of both *E. coli* and *Cl. perfringens* was not supported by these particular low molecular weight hydrolysates ($p < 0.05$) (and significantly more decreased than the corresponding growth on galactose and FOS) as it was low on galactan – the only exception being the growth on PG5 which for the *E. coli* was the same as that obtained on galactose, but which for *Cl. perfringens* was lower than that on galactose, but higher than on FOS (Fig. 8). Whether PG5 is as promising a prebiotic agent as some of the other produced fractions requires further investigation, e.g. by incubation with mixed microbiota and assessment of whether competition and/or cross-feeding phenomena influence the growth of the pathogen. Galactan, as well as, samples PG2 and PG3 (3 kDa < PG2 < 10 kDa, 60 min, E/S 0.03%; and PG3 < 10 kDa, 15 min, E/S 0.3%) did not appear to support the growth of *Cl. perfringens*. Neither did of these substrates support the growth of very high levels of any of the three "probiotic" strains, and the growth of *E. coli* was also much lower than that achieved with galactose.

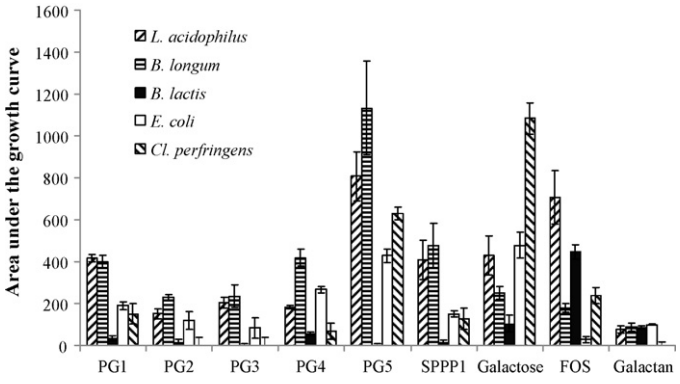

Fig. 8. Differential growth of bacterial strains on *E. nidulans* endo-1,4-β-galactanase catalyzed hydrolysis products from potato galactan and SPPP (please see text for details); galactose and fructo-oligosaccharides (FOS) were used as controls; growth responses for all the 9 substrates is shown for a substrate concentration 10 g/L for all bacterial strains. Data are given as average values of 3–6 growth assay replicates and shown ± S.D. (for the one-way ANOVA of the bacterial growth the pooled S.D.s were: *L. acidophilus*: 74.6; *B. longum* 51.7; *B. lactis* 17.2; *E. coli*: 31.3; *Cl. perfringens*: 41.3; for the total ANOVA (level: 45) the pooled S.D. was: 50.1).

Even though the growth stimulation of *E. coli* by these substrates was higher or at least the same level as that obtained with FOS (Fig. 8) further investigation concerning the structural traits of the compounds present in these endo-β-1,4-galactanase hydrolyzed fractions and identification of the presumed key potentially prebiotic compounds is warranted. An additional point is that the presence of galactose would most likely not be important *in vivo* since the human organism would absorb the galactose before it reaches the colon. Although the detailed data analysis revealed that unhydrolyzed galactan stimulated the growth of *B. lactis* to the same extent as neat galactose, hardly any growth of *B. lactis* was obtained with any of the substrates, except with FOS (Fig. 8).

In summary, contemplation of the bioscreen data showed that among the tested samples the lower molecular weight fractions exhibited more and various potentially prebiotic features.

Whether the observed low or no growth supporting effects on *Cl. perfringens* have any significance in the genuine competition among the bacteria in the mixed microbiota in the gut deserve further investigation.

An evaluation of the enzymes annotated for the sequenced genomes for different species within *Bifidobacterium* available via the CAZy database [25] shows that bifidobacteria are generally capable of expressing β-galactosidase (EC 3.2.1.23),

α-galactosidase (EC 3.2.1.22), endo-β-1,6-galactanase (EC 3.2.1.164), exo-β-1,3-galactosidase (EC 3.2.1.145), α-1,3-galactosidase (EC 3.2.1.-) and endo-β-1,4-galactanase (EC 3.2.1.89) [31]. Endo-β-1,4-galactanase (GH53) can be expressed by *B. dentium* (Bd1), *B. longum* (DJO10A and NCC2705), *B. longum* subsp. *infantis* (157F and JCM1222), *B. longum* subsp. *longum* (F8, BBMN68, JDM301 and JCM1217) and *Lactobacillus reuteri* (DSM20016 and JCM1112) but a gene sequence for this enzyme activity has not been identified in any of the other genome sequenced *Bifidobacterium* and *Lactobacillus* species. Exo-β-1,3-galactosidase (GH43) has been annotated for more than 15 different bifidobacteria but only in four lactobacilli. Endo-β-1,6-galactanase (GH5 and GH30) were annotated for several bifidobacteria (>12) but only in two lactobacilli. Lastly, α-1,3-galactosidase (GH110) was annotated for *B. bifidum* (PRL2010 and S17) but not found in lactobacilli. Different species of *Bacteroidetes* and *Firmicutes* were also reported to be able to express some of these enzymes [25], but gene stretches encoding all the enzyme activities were not present in all the available genomes. The data suggest that especially *B. longum* may express particularly high activities of some of these enzymes and this could give a selective advantage in utilizing the β-1,4-linked galactans and β-1,4-linked-oligomers.

The present work was conducted using single cultures, but the results obtained are in good accord with the amply reported prebiotic effects of galacto-oligosaccharides [32,33]. The data also agree with recent data from a human study (a double-blind, crossover, placebo-controlled intervention study with 59 human volunteers), which have shown that certain enzymatically synthesized β-galacto-oligosaccharides, consumed at levels of 7 g/day, significantly increase the bifidobacteria ratio compared with a commercial galacto-oligosaccharide ($p < 0.05$) [34]. It has also been shown that these enzymatically synthesized β-galacto-oligosaccharides have potential in alleviating symptoms of irritable bowel syndrome and in preventing the incidence and symptoms of travellers' diarrhea [35,36]. The latter bifidogenic β-galacto-oligosaccharides were synthesized by use of β-galactosidase enzymes originating from *B. bifidum NCIMB* 41171 [34–36]. The data clearly suggest that β-galacto-oligosaccharides whether obtained via enzyme catalyzed hydrolysis of longer chain β-galactan structures or via β-galactosidase catalyzed synthesis possess promising beneficial properties in relation to gut health. The available data also suggest that different sub-structures of these β-galactosides, e.g. certain molecular sizes and/or branching patterns, exhibit differential prebiotic effects. At present, there is a severe lack of knowledge about the specific carbohydrate structural effects and about the interactions among different "probiotic" and pathogenic strains *in vivo*; it can be speculated that the different human gut bacteria respond differently to compounds and metabolites produced by other bacteria and thus interact highly differently to cross-feeding phenomena. Any data on this would provide a significantly improved foundation for tailoring of carbohydrate structures for gut health.

3.8. Conclusions

A very active endo-1,4-β-galactanase from *E. nidulans* expressed in *P. pastoris* has successfully been produced in a 5 L scale fermentation and purified with a high yield (1.6 g/L). The enzyme was used to design oligosaccharides from high molecular weight β-1,4-galactan-rich potato fibers (SPPP) as well as from potato galactan in membrane reactors. A range of these products were demonstrated to have significant prebiotic potential on single cell cultures of gut microbes. The results of this present work have provided an additional positive foundation for biocatalytic design of β-galacto-oligosaccharides with putative health effects. The work has also shown a direction for potential valorization of the potato pulp byproduct stream.

Acknowledgments

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10.4 The binding of zinc ions to *Emericella nidulans* endo-ß-1,4-galactanase is essential for crystal formation

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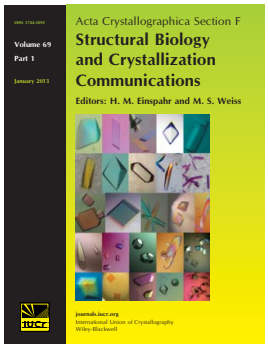

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The binding of zinc ions to *Emericella nidulans* endo-β-1,4-galactanase is essential for crystal formation

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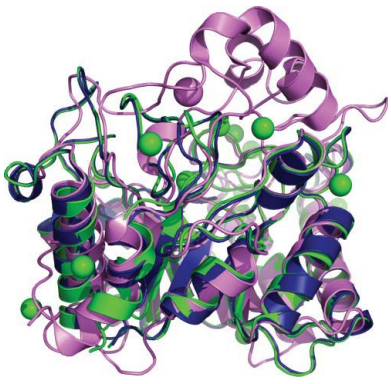
A novel *Emericella nidulans* endo-β-1,4-galactanase (*En*GAL) demonstrates a strong capacity to generate high levels of very potent prebiotic oligosaccharides from potato pulp, a by-product of the agricultural potato-starch industry. *En*GAL belongs to glycoside hydrolase family 53 and shows high (72.5%) sequence identity to an endo-β-1,4-galactanase from *Aspergillus aculeatus*. Diffraction data extending to 2.0 Å resolution were collected from a crystal of *En*GAL grown from conditions containing 0.2 *M* zinc acetate. The crystal structure showed a high similarity between *En*GAL and other endo-β-1,4-galactanases belonging to GH53. It also revealed 15 zinc ions bound to the protein, one of which is located in the active site, where it is coordinated by residues Glu136 and Glu246 which comprise the catalytic machinery. The majority of the zinc ions are located on the surface of the enzyme, in some cases with side chains from two different molecules as ligands, thus explaining why the presence of zinc ions was essential for crystallization.

1. Introduction

Enzymatically solubilized polysaccharides from potato pulp have been demonstrated to exert potentially beneficial properties as dietary fibre and prebiotics in both *in vitro* (human faecal fermentations) and *in vivo* (rat and human) studies (Thomassen, Vigsnaes *et al.*, 2011). Recently, high yields of pectinaceous polysaccharides with high molecular weights have been released by a one-step enzymatic process using the enzymes pectin lyase and polygalacturonase from *Emericella nidulans* (a sexual anamorph of *Aspergillus nidulans*; Thomassen, Larsen *et al.*, 2011). The soluble, high-molecular-weight potato-pulp polysaccharides showed very promising prebiotic properties. Further hydrolysis of these high-molecular-weight polysaccharides with *E. nidulans* endo-β-1,4 galactanase expressed in *Pichia pastoris* showed that the high-molecular-weight potato β-1,4-galactan could be further enzymatically degraded. The resulting product, a lower and narrower molecular-weight galactan, exhibits even more potent prebiotic effects on human gut microbiota. *En*GAL shows optimal activity at 322 K and pH 5.0. Its half-life is 57 min at 313 K, 27 min at 322 K and 10.5 min at 333 K. On the addition of divalent metal ions (Ca^{2+}, Mn^{2+} and Zn^{2+}) a minor increase in activity was observed (Michalak *et al.*, 2012).

Sequence comparison showed that this endo-β-galactanase (*En*GAL) belongs to CAZy family GH53 (Cantarel *et al.*, 2009) and is part of the largest glycoside hydrolase clan (clan A; Supplementary Table S1[1]). It acts with retention of the configuration at the anomeric position in a double-displacement mechanism (Jenkins *et al.*, 1995; Henrissat *et al.*, 1995). *En*GAL shows 72.5% sequence identity to the endo-β-1,4-galactanase from *A. aculeatus* (AAGAL), the structure of which has been determined at two temperatures: 293 and 100 K (PDB entries 1fhl and 1fob; Ryttersgaard *et al.*, 2002). Le Nours *et al.* (2003) determined the structures of two other fungal endo-β-1,4-galactanases with higher temperature optima, one from *Humicola insolens* (HIGAL; PDB entry 1hjq) and the other from *Myceliophthora thermophilia* (MTGAL; PDB entries 1hjs and 1hju). The structure is also known of an endo-β-1,4-galactanase from *Bacillus licheniformis*



[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: SW5064).

Table 1
*En*GAL gene construct and enzyme production.

Source organism	*A. nidulans* FGSC A4 (*E. nidulans*)
DNA source	Chromosomal DNA
Forward primer	GAAAGCACGTGATATGATTCTCTCCTCCCTCTTA
Reverse primer	TACAGTCTAGAAGATCACCAAAAACACTTATACTT
Cloning and expression vector	pPICZαC
Expression host	*P. pastoris*
Complete amino-acid sequence of the construct produced	MILSSLLPLSLVTLTSAALTYRGADISSLLIEEDSGVAYK-NLNGETQAFELILANNGVNSIRQRIWVNPSDGSYNL-EYNLELAKRVQDAGMSVYLDLHLSDTWADPGDQA-TPSGWSTTDIDTLAWQVYNYTLDVCNTFAENNVAV-EIVSIGNEIRNGLLHPLGSTDHYDNIARLLHSGAWG-VKDSSLSTTPKILFHLDNGWDWDAQKYFYDTVLAT-GTLLSTDFDLIGVSYYPFYNADATLSSLKTSLTNLKS-NYGKNVLVVETDWPVQCSSPEYAFPSDLSSIPFSADG-QETFLGRLADTLEDVGGVGIYYWEPGWVDNAGLG-SSCEDNLMVDWRDRTVRESISVFGDLHHHHHH

(BLGAL) both in the apo form and as oligosaccharide complexes (PDB entries 1r8l, 1ur0, 1ur4, 2ccr, 2gft and 2j74; Ryttersgaard *et al.*, 2004; Le Nours *et al.*, 2009). In order to provide a structural background for the functional analysis of *En*GAL in relation to the other enzymes from family GH53, structure determination of *En*GAL was initiated.

2. Materials and methods

2.1. Macromolecule production

The endo-β-1,4-galactanase gene AN5727.2 (GenBank ABF50874.1; UniProtKB/SwissProt Q5B153) was obtained from the Fungal Genetics Stock Center (http://www.fgsc.net). It had been cloned earlier as the gene construct shown in Table 1 (Bauer *et al.*, 2006). The expression in *P. pastoris*, characterization and purification of *En*GAL have been described in detail elsewhere (Silva *et al.*, 2011; Michalak *et al.*, 2012). The gene construct included a C-terminal 6×His tag. A His-tag IMAC column was used for purification using a binding buffer consisting of 0.04 *M* EPPS pH 8.0, 0.5 *M* NaCl and subsequent elution with 0.04 *M* EPPS, 0.5 *M* NaCl, 0.5 *M* imidazole. The imidazole was removed by gel filtration (PD10 column; GE Healthcare Life Sciences) with 0.04 *M* EPPS pH 8.0, 0.5 *M* NaCl. The protein was stored at 253 K with 25%(*v*/*v*) glycerol added.

2.2. Crystallization

The *En*GAL solution was concentrated to 23.9 mg ml^{-1} on a cellulose membrane and was subsequently spun for 10 min at 10 000 rev min^{-1} to remove higher molecular-weight aggregates. The supernatant was used to set up MRC 2 Well sitting-drop trays with an Oryx 8 Crystallization Robot (Douglas Instruments Ltd). Crystallization trials were carried out with the Index HT, JCSG+ and PACT screens at room temperature using the same protein buffer (0.04 *M* EPPS pH 8.0, 0.5 *M* NaCl) for all experiments. The volume of the 1:1 ratio drops was 300 nl and the volume of the reservoir was 100 µl. A box-shaped single crystal with approximate dimensions of 50 × 50 × 150 µm appeared after five months in a condition consisting of 0.2 *M* zinc acetate, 0.1 *M* imidazole pH 8.0, 20%(*w*/*v*) PEG 3000. None of the other screening conditions gave any crystalline material. The crystal was cryoprotected with mother liquor and cryoprotectant solution (16% glycerol, 16% ethylene glycol, 18% sucrose, 4% glucose) in a 1:1 ratio and flash-cooled in liquid nitrogen.

Table 2
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	ID23-2 beamline, ESRF
Wavelength (Å)	0.8726
Temperature (K)	100
Detector	225 mm MAR Mosaic
Crystal-to-detector distance (mm)	196.36
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	3
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 62.90, b = 72.37, c = 98.38
Mosaicity (°)	0.2
Resolution range (Å)	47–2.0 (2.12–2.00)
Total No. of reflections	226112 (34730)
No. of unique reflections	58261 (9139)
Completeness (%)	99.4 (96.5)
Multiplicity	3.9 (3.8)
$\langle I/\sigma(I)\rangle$	9.3 (2.0)
$R_{\rm r.i.m.}$ (%)	14.4 (76.8)
Overall B factor from Wilson plot (Å^2)	20.9

Table 3
Structure-refinement statistics for *En*GAL.

Resolution range (Å)	47–2.0 (2.11–2.00)
Completeness (%)	99.5
No. of reflections, working set	58202
No. of reflections, test set	2912
Final $R_{\rm cryst}$	0.159
Final $R_{\rm free}$	0.194
Cruickshank DPI	0.1
No. of non-H atoms	5357
Protein	5092
Glycosylation	14
Cations	15
Ligands (acetate, imidazole, glycerol)	55
Water molecules	181
R.m.s. deviations	
Bonds (Å)	0.012
Angles (°)	1.312
Average B factors (Å^2)	
Protein	22.3
Cations	29.9
Ligands	43.4
Water molecules	31.7
Ramachandran plot	
Favoured regions (%)	96.4
Additionally allowed (%)	2.7
Outliers (%)	0.6

2.3. Data collection and processing

X-ray diffraction data were collected on ESRF beamline ID23-2 (Flot *et al.*, 2010) from the crystal cooled to 100 K. The strategy devised by *EDNA* (Incardona *et al.*, 2009) was used in the data collection, which was performed with radiation of wavelength 0.8726 Å. The crystal diffracted to 2.0 Å resolution. Processing of the 180 images with *XDS* (Kabsch, 2010) showed that the crystal belonged to the orthorhombic space group $P2_12_12_1$. The intensities were scaled with *XSCALE* (Kabsch, 2010). Details of the data collection and data processing are presented in Table 2.

2.4. Structure solution and refinement

The structure of *En*GAL was determined by the molecular-replacement method using *A. aculeatus* endo-β-1,4-galactanase (AAGAL; PDB entry 1fob; Ryttersgaard *et al.*, 2002) as a search model. Employing *PHENIX* (Adams *et al.*, 2010), *phenix.automr* gave convincing *Z*-scores (RFZ 29.6, TFZ 42.2) and a positive log-likelihood gain for a solution with one molecule per asymmetric unit, corresponding to a solvent content of 60%. The molecular-replacement solution was adapted to the correct sequence, covering amino

acids 18–352, by *phenix.autobuild* and manual building. The difference map showed 15 peaks close to negatively charged side chains, which were consistent with bound metal ions. In native AAGAL and BLGAL (Ryttersgaard *et al.*, 2002, 2004) crystallized in the presence of calcium ions, only one calcium ion could be located in each structure. In the present case the crystallization conditions contained 0.2 *M* zinc acetate, which made zinc ions the most likely candidate. At the wavelength used for data collection f'' for calcium is significantly smaller (less than ∼25%) than f'' for zinc. The anomalous difference map revealed fairly large peaks at all proposed metal sites, which verified them as zinc ions that could be refined at full occupancy. The difference density revealed *N*-acetylglucosamine (GluNAc) bound to Asn112. The final model also includes two residues from the His tag, which were modelled as Ala. Structure refinement was carried out with *phenix.refine* (see Table 3 for statistics). The Ramachandran plot was generated with *MolProbity* (Chen *et al.*, 2010).

3. Results and discussion

3.1. Overall structure

The overall fold of *En*GAL is essentially identical to the previously observed $(\beta/\alpha)_8$ barrel of the other three fungal galactanases belonging to GH53. This fold is highly conserved and *En*GAL has an all-atom r.m.s.d. of 0.374 Å to its closest relative AAGAL. The cysteine bridge between Cys253 and Cys310 is conserved in the fungal enzymes. Among the four structurally characterized β-1,4-galactanases, BLGAL is the enzyme that deviates most from the fungal galactanases, with an additional loop at the N-terminus, exhibiting an r.m.s.d. of 0.972 Å to *En*GAL. Fig. 1 shows an overlay of *En*GAL with the closest (AAGAL) and the least similar (BLGAL) endo-galactanase structures. The sequence differences between *En*GAL and the other fungal galactanases are predominantly found in the regions involved in ion binding (see below) and in residues 318–334

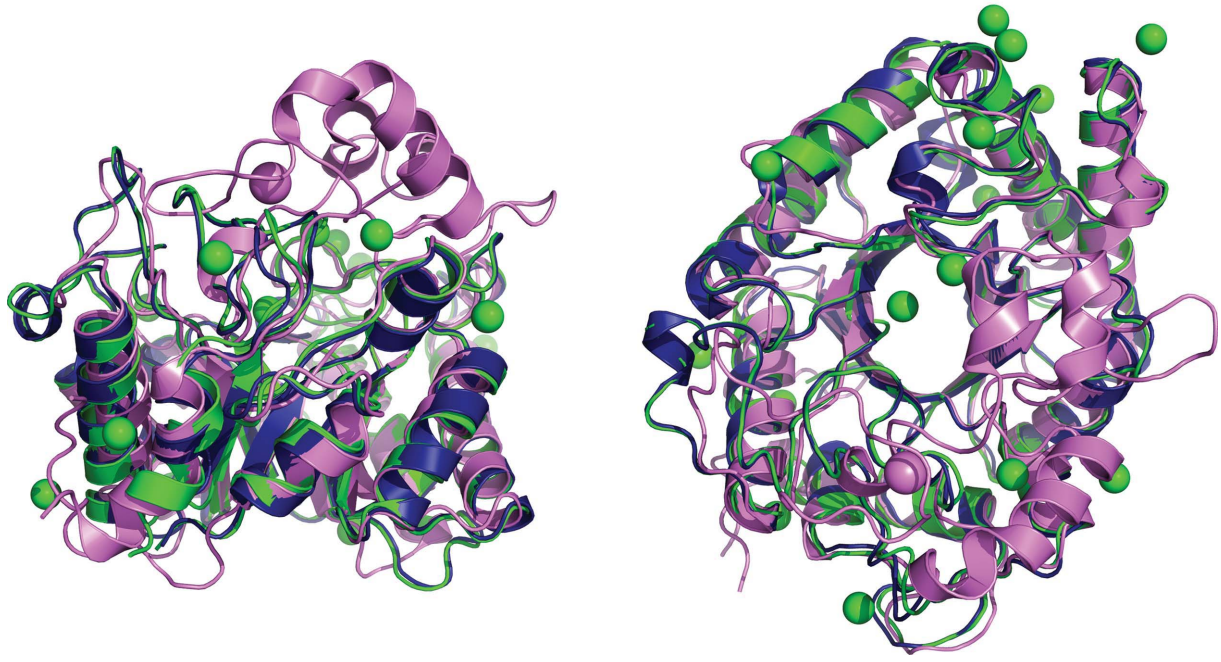
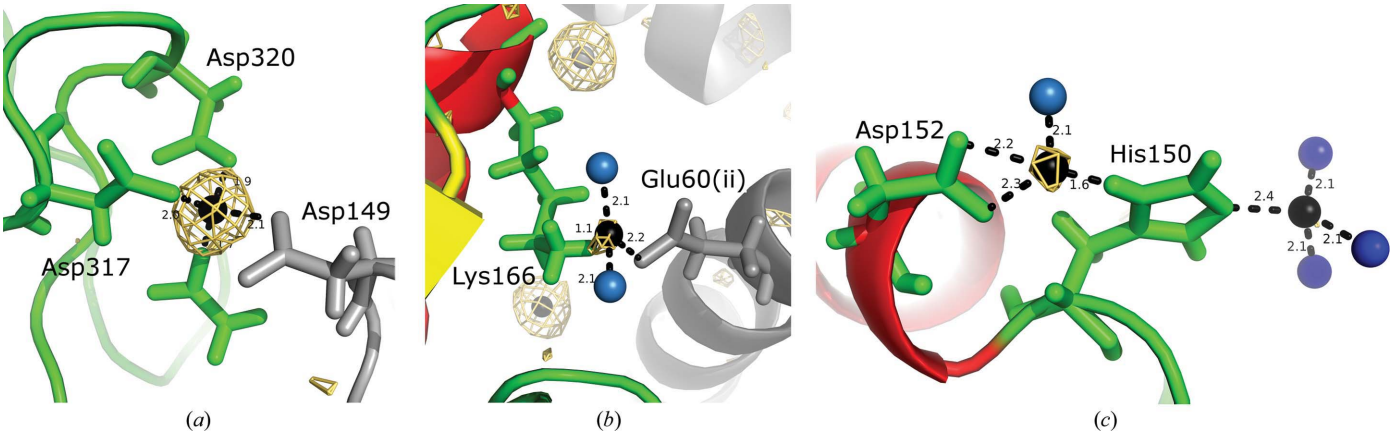


Figure 1
Overlay of endo-β-1,4-galactanases from family GH53: *En*GAL (in green) superimposed on the closest relative AAGAL (PDB entry 1fob; blue; r.m.s.d. 0.374 Å) and the least similar BLGAL (PDB entry 1r8l; violet; r.m.s.d. 0.972 Å). The zinc ions bound to *En*GAL are illustrated as green spheres. The violet sphere is the calcium ion in BLGAL. All figures were generated with *PyMOL* (DeLano, 2002).



(*a*) (*b*) (*c*)

Figure 2
Illustration of selected zinc sites in *En*GAL, with the anomalous difference map in yellow contoured at the 5σ level. (*a*) Zinc ion 402 coordinated by three amino acids from two molecules; (*b*) zinc ion 405 coordinated by two side chains from two molecules and two water molecules; (*c*) His150 bridging zinc ions 404 and 414.

of the C-terminus, where BLGAL possesses an additional α-helix (Fig. 1 and Supplementary Table S1). The GluNAc *N*-glycosylation at Asn112, analogous to Asn111 in MTGAL and HIGAL, is consistent with previous observations for proteins expressed in *P. pastoris* (Zou *et al.*, 2013). The active site of *En*GAL with the conserved catalytic machinery comprised of Glu136 and Glu246 contains a zinc ion (Supplementary Fig. S3).

3.2. Zinc-binding sites

The anomalous difference map and analysis of the surroundings of the peaks identified 15 zinc ions bound to *En*GAL. The coordination of the ions is in accordance with the results obtained previously by Harding (2004) for protein-bound zinc ions. Although the coordination geometry of the zinc ions (Supplementary Table S2) is predominantly tetrahedral, there are also ions with octahedral and trigonal bipyramidal coordination. Only Zn407 is found in the interior of the protein, coordinated by the two catalytic residues Glu136 and Glu246 (Supplementary Fig. S3). The surface-bound zinc ions in the crystal structure of *En*GAL display variety in their coordination environment, as illustrated by a few examples. Fig. 2(*a*) shows the tetrahedral coordination of Zn402 by four carboxylate groups: Asp317 and Asp320 from its parent protein, Asp149 from a symmetry-related molecule and an acetate group. Fig. 2(*b*) shows how Zn405 is coordinated by two water molecules and the side chains of Glu60 and Lys166, again from two different molecules in the unit cell. The side chain of Lys166 is neutral owing to the high pH for crystallization and to the proximity to the zinc ion. A similar role of bridging symmetry-related molecules is found for five of the other zinc ions (Zn401, Zn408, Zn409, Zn410 and Zn412). Finally, Fig. 2(*c*) depicts how the His150 side chain links two zinc-ion sites (Zn404 and Zn414) from the same molecule.

3.3. The role of the zinc ions

It is noteworthy that it was only possible to obtain crystals of *En*GAL at a high pH in the presence of 0.2 *M* zinc acetate. The structure analysis showed that about half of the bound zinc ions bridge the molecules in the unit cell (Supplementary Fig. S4), which explains why the presence of zinc ions was imperative for the crystallization of *En*GAL. A search of the PDB revealed several similar examples of proteins with metal ions from the crystallization conditions bound to the surface (Zn in PDB entry 1aol, Fass *et al.*, 1997; Cu in PDB entry 3zud, Quinlan *et al.*, 2011). Recently, Cha *et al.* (2012) conducted a thorough study of zinc-ion binding to proteins and showed how an apparently nonspecific binding to the surface of proteins could be used to provide experimental phases. Crystallization of a protein at high pH in the presence of 0.003–0.3 *M* zinc ions can facilitate crystal formation by stabilizing intermolecular interactions, and the anomalous scattering from the zinc can aid the structure determination.

An interesting question remains: why does *En*GAL bind zinc ions to an extent not seen for the other fungal galactanases? The calculated pI values for the fungal galactanases span from 4.0 (AAGAL) to 6.0 (BLGAL, MTGAL, HIGAL) and is 4.2 for *En*GAL, the pI of which has been experimentally determined to be below 3.5. Although the overall negative charge does not differ greatly from the charge of AAGAL, a depiction of the surface charge of *En*GAL reveals several negatively charged patches suitable for possible metal-ion binding (Fig. 3). Furthermore, the 15 zinc ions compensate the 29 negative

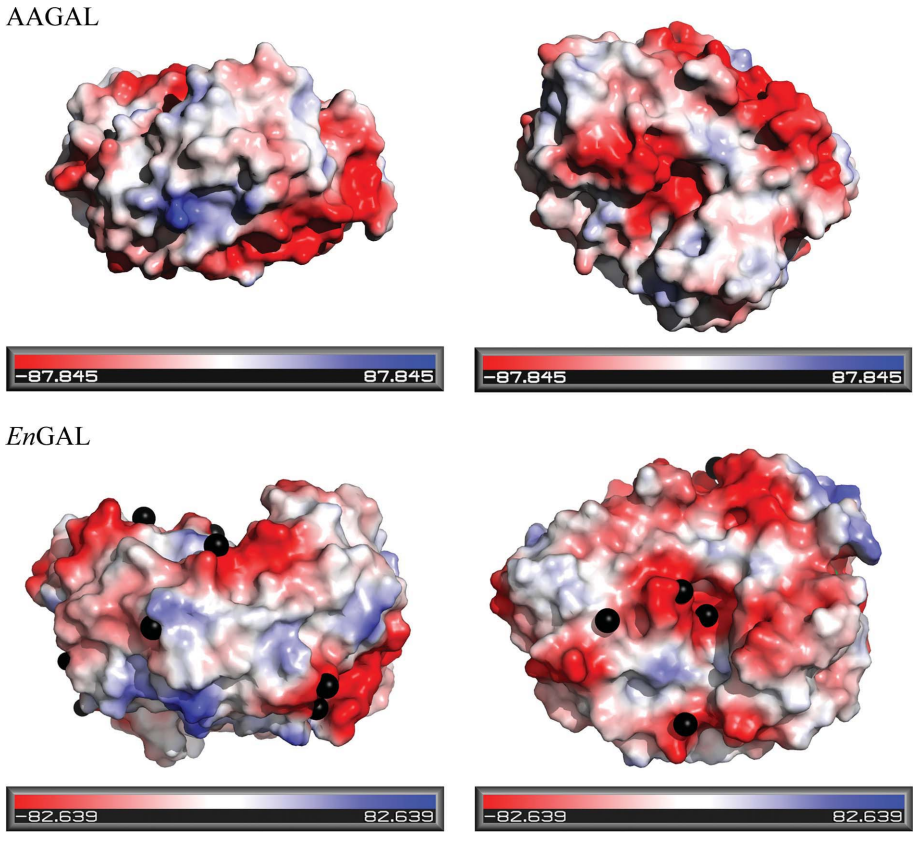


Figure 3
Relative surface charges plotted at neutral pH for AAGAL and *En*GAL without the contribution from the bound metal ions (shown as black spheres).

charges of the galactanase polyanion, which favours crystallization in addition to bridging different molecules in the unit cell. The non-covalent cross-linking of molecules by zinc ions facilitates crystallization and acts similarly to glutaraldehyde-linking (Wine *et al.*, 2007) and protein-engineering approaches (Derewenda & Vekilov, 2006).

*En*GAL shows increased activity in the presence of divalent metal ions (Michalak *et al.*, 2012). The structure did not suggest any catalytic role for the zinc ions, so the likely explanation for the increased activity is that the bound zinc ions exert a stabilizing effect on the structure and neutralize the surface charge, which could favour interactions with the neutral substrate.

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